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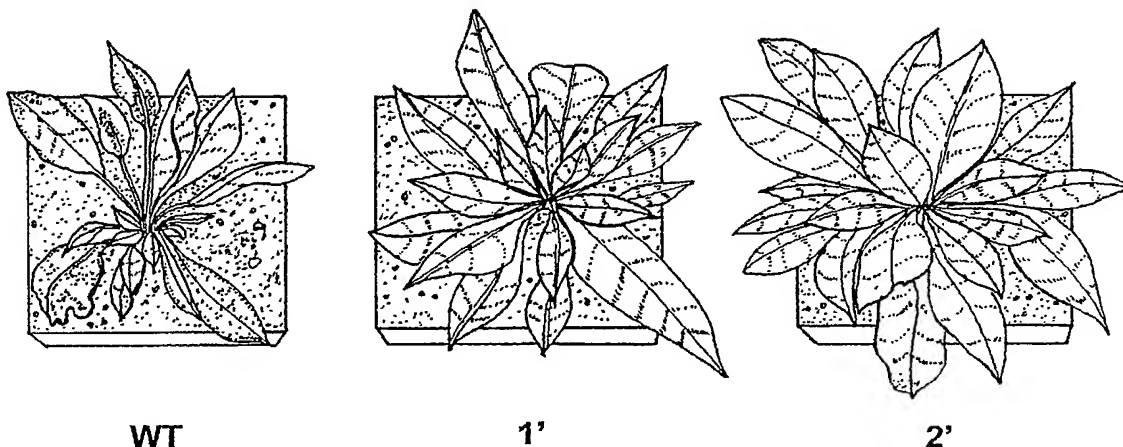
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(54) Title: STRESS-RESISTANT OVERSIZED TRANSGENIC PLANTS CAPABLE OF GROWING IN SALINIZED SOIL



WT

1'

2'

(57) Abstract: A stress resistant, oversized, transgenic plant capable of growing in salinized media comprising a polynucleotide sequence causing upregulated express of vacuolar pyrophosphatase. Further disclosed, is the seed produced by such transgenic plants which comprises such polynucleotide sequence, and progeny plants grown from such seed.

WO 01/33945 A1

**STRESS-RESISTANT OVERSIZED TRANSGENIC PLANTS  
CAPABLE OF GROWING IN SALINIZED SOIL**

**RELATED APPLICATION(S)**

This application claims the benefit of U.S. Provisional Application No. 60/164,808, entitled "Proton Transporters and Uses in Plants" by Roberto Gaxiola, filed November 10, 1999, U.S. Patent Application No. 09/644,039 entitled "Proton Transporters and Uses in Plants" by Roberto Gaxiola, filed August 22, 2000, and U.S. Provisional Application No. 60/226223, entitled "Drought/Freeze Resistant Transgenic Plants" by Roberto Gaxiola, filed August 18, 2000, the entire teachings of all of which are incorporated herein by reference.

**GOVERNMENT SUPPORT**

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**BACKGROUND OF THE INVENTION**

1. **Field of the Invention**

The present invention relates to genetically-altered plants that are hardy with respect to environmental stresses, such as drought and/or freezing, oversized with

respect to vegetative and/or sexual structure (as compared to their normal phenotypic counterparts), and capable of growing in media of high salinity.

2. Background of the Related Art

The prospects for feeding humanity as we enter the new millennium are formidable. Given the every increasing world population, it remains a major goal of agricultural research to improve crop yield. It also is a major goal of horticultural research to develop non-crop plants which are hardier, such as ornamental plants, grasses, shrubs, and other plants found useful or pleasing to man.

Until recently crop and horticultural improvements depended on selective breeding of plants having desirable characteristics. Such selective breeding techniques, however, were often less than desirable as many plants have within them heterogenous genetic complements that do not result in identical desirable traits of their parents.

Advances in molecular biology have allowed mankind to manipulate the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants with increased pest resistance, plants that are capable of expressing pharmaceuticals and other chemicals, and plants that express beneficial traits. Advantageously such plants not only contain a gene of interest, but remain fertile.

One particular area of interest of late in plant sciences has been the development of plants with improved stress resistance. In general, plants possess and maintain adaptive mechanisms to ensure survival during periods of adverse environmental conditions. Two commons stresses that plants commonly encounter are freezing and drought, both of which are associated with cellular dehydration. It is known that certain plants contain genes turned on by exposure to cold or prolonged periods of dehydration that encode for products that are directly or indirectly responsible for providing greater resistance to drought and/or freeze than many of their counterparts. A number of genes responsive to heat and water stress have now been characterized (See, e.g., U.S. Pat. Nos. 5,837,545, 5,071,962, 4,707,359). These genes are believed by many to produce certain proteins, such as "Water Stress Proteins", that are postulated to aid the plants survival. For example, certain plants exposed to stress conditions produce a hormone called abscisic

acid (ABA) which helps plants close their stomata, thereby reducing the severity of the stress. Unfortunately, ABA is known to inhibit the formation of new leaves, to cause flowers and fruit to drop off, and to lead to a reduction in yield.

Most tropical plants are not believed to have evolved the ability to tolerate prolonged drought and/or freezing. Conversely, many temperate plants are known to have developed at least some ability to tolerate such conditions. The productivity of plant varieties in dry conditions, and after freeze, differ dramatically. For example, tobacco (*Nicotiana* spp.) produces fresh leaves that are highly sensitive to drought, and cannot be produced commercially in areas with a limited water supply and high degree of evaporation. In contrast to water stress, very little is known about proteins and genes which participate in freezing tolerance. However, it has been hypothesized that a major component of freeze tolerance may involve tolerance to dehydration (See, *e.g.*, Yelenosky, G.C., Guy, L. (1989) *Plant Physiol.* 89: 444-451).

Another particular area of interest of late has been the development of plants with improved abilities to grow in salinized soil. Salinization of soil occurs when water supplies contain dissolved salt. Upon evaporation of water from such supplies, salts gradually accumulate in the soil. The progressive salinization of irrigated land compromises the future of agriculture in many of the most productive areas of our planet (Serrano, R., *et al.*, *Crit. Rev. Plant Sci.*, 13:121-138 (1994)). For example, arid regions offer optimal photoperiod and temperature conditions for the growth of most crops, but suboptimal rainfall. Artificial irrigation has solved the problem only in the short term as it has been found that soils in such environments frequently are rapidly salinized. To grow in salinized environments, plants must maintain a much lower ratio of  $\text{Na}^+/\text{K}^+$  in their cytoplasm than that present in the soil, preventing the growth of a number of plants, including food crops.

Physiological studies suggest that salt exclusion in the root, and/or salt sequestration in the leaf cell vacuoles, are critical determinants for salt tolerance (Kirsch, M., *et al.*, *Plant Mol. Biol.*, 32:543-547 (1996)). Toxic concentrations of sodium chloride (NaCl) build up first in the fully expanded leaves where NaCl is compartmentalized in the vacuoles. Only after their loading capacity is surpassed, do the cytosolic and apoplasmic concentrations reach toxic levels, ultimately leading to loss of turgor, *ergo* plant death. It has been suggested that hyperacidification of the vacuolar lumen via the V-ATPase

provides extra protons required for a  $\text{Na}^+/\text{H}^+$  exchange-activity leading to the detoxification of the cytosol (Tsiantis, M.S., *et al.*, *Plant J.*, 9:729-736 (1996)). Salt stress is known to increase both ATP- and pyrophosphate (PPi)-dependent  $\text{H}^+$  transport in tonoplast vesicles of, for example, sunflower seedling roots. Salt treatments also induce an amiloride-sensitive  $\text{Na}^+/\text{H}^+$  exchange activity (Ballesteros, E., *et al.*, *Physiologia Plantarum*, 99:328-334 (1997)). In the halophyte *Mesembryanthemum crystallinum*, high NaCl stimulates the activities of both the vacuolar  $\text{H}^+$ -ATPase (V-ATPase) and a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter in leaf cells.

Yet another area of agricultural interest is to improve the yield of crop plants and to improve the aesthetic qualities of certain decorative plants. The yield of a plant crop, and the aesthetics of certain decorative plants, may be improved by growing plants that are larger than the wild-type plant in vegetative and/or reproductive structure. It is known in the art that certain growth factors may be used to increase plant and/or plant flower size. Unfortunately, application of such growth factors is costly and time consuming.

A need, therefore, exists for plants having improved stress resistance to drought and/or freeze, possessing larger size attributes than wild-type counterpart varieties, and having increased tolerance to salt in the soil in which they are growing.

### **SUMMARY OF THE INVENTION**

The present invention discloses a transgenic plant having upregulated expression of vacuolar pyrophosphatase. It has been found that plants displaying such upregulated activity are generally larger than wild-type counterparts, demonstrate improved stress resistance to drought and/or freeze, and have increased tolerance to salt in the media in which they are growing.

Any suitable exogenous nucleic acid molecule which alters expression of vacuolar pyrophosphatase in the plant can be used to transform the transgenic plants in accord with the present invention. The exogenous nucleic acid can comprise nucleic acid that encodes a vacuolar pyrophosphatase protein (an exogenous vacuolar pyrophosphatase), such as AVP1, a functional portion thereof (peptide, polypeptide), or a homologue thereof, and/or nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced. By

“exogenous nucleic acid” it is meant a nucleic acid from a source other than the plant cell into which it is introduced, or into a plant or plant part from which the transgenic part was produced. The exogenous nucleic acid used for transformation can be RNA or DNA, (*e.g.*, cDNA, genomic DNA). In addition, the exogenous nucleic acid can be circular or linear, double-stranded or single-stranded molecules. Single-stranded nucleic acid can be the sense strand or the anti-sense strand. By a “functional portion” of a nucleic acid that encodes a vacuolar pyrophosphatase protein it is meant a portion of the nucleic acid that encodes a protein or polypeptide which retains a functional characteristic of a vacuolar pyrophosphatase protein. In a particular embodiment, the nucleic acid encodes AVP1, a functional portion or a homologue thereof.

Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced includes regulatory sequences (*e.g.*, inducible, constitutive) which function in plants and antisense nucleic acid. Examples of regulatory sequences include promoters, enhancers and/or suppressors of vacuolar pyrophosphatase. The nucleic acid can also include, for example, polyadenylation site, reporter gene and/or intron sequences and the like whose presence may not be necessary for function or expression of the nucleic acid but can provide improved expression and/or function of the nucleic acid by affecting, for example, transcription and/or stability (*e.g.*, of mRNA). Such elements can be included in the nucleic acid molecule to obtain optimal performance of the nucleic acid.

The nucleic acid for use in the present invention can be obtained from a variety of sources using known methods. For example, the nucleic acid encoding a vacuolar pyrophosphatase (*e.g.*, AVP1) for use in the present invention can be derived from a natural source, such as tobacco, bacteria, tomato or corn. In one embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that corresponds to a wild type of the transgenic plant. In another embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that does not correspond to a wild type of the transgenic plant. Nucleic acid that alters expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced (*e.g.*, regulatory sequence) can also be chemically synthesized, recombinantly produced and/or obtained from commercial sources.

A variety of methods for introducing the nucleic acid of the present invention into plants are known to those of skill in the art. For example, Agrobacterium-mediated plant transformation, particle bombardment, microparticle bombardment (*e.g.*, U.S. Patent No. 4,945,050; U.S. Patent No. 5,100,792) protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos can be used. The exogenous nucleic acid can be introduced into any suitable cell(s) of the plant, such a root cell(s), stem cell(s) and/or leaf cell(s) of the plant.

Any suitable plant can be used to produce the transgenic plants of the present invention. For example, tomato, corn, tobacco, rice, sorghum, cucumber, lettuce, turf grass, ornamental (*e.g.*, larger flowers, larger leaves) and legume plants can be transformed as described herein to produce the transgenic plants of the present invention. In addition, the transgenic plants of the present invention can be grown in any medium which supports plant growth such as soil or water (hydroponically).

A transgenic plant of the present invention is preferably tolerant to high salt concentrations in soil. By the term "salt" it is meant to include any salt, that is a compound formed when hydrogen of an acid is replaced by a metal or its equivalent, and includes, without limitation, salts comprising monovalent and divalent toxic cations, NaCl, KCl, CaCl<sub>2</sub>, MgCl, CdCl, ZnCl, and sulfide salts.

Salt tolerance may be introduced into a plant of the present invention by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that expression is upregulated. Any suitable vacuolar pyrophosphatase, several of which have been cloned, can be used in the compositions and methods of the present invention (*e.g.*, Sarasian, Z., *et al.*, *Proc. Natl. Acad. Sci., USA*, 89:1775-1779 (1992); Jenslerchl, *et al.*, *Molec. Biol.*, 29: 833-840 (1995); Kim, Y., *et al.*, *Plant Physiol.*, 106:375-382 (1994)). In a particular embodiment, the present invention relates to a transgenic plant which is tolerant to salt comprising an exogenous nucleic acid construct which is designed to overexpress AVP1 (Sarasian, Z., *et al.*, *Proc. Natl. Acad. Sci., USA*, 89:1775-1779 (1992)). Transformation of the plant cells may be carried out in a whole plant, seeds, leaves, roots or any other plant part. Such transgenic plants are preferably altered such that they grow in a concentration of salt that inhibits growth of a corresponding non-transgenic plant. Transgenic progeny of the transgenic plants, seeds produced by the transgenic plant and progeny transgenic plants

grown from the transgenic seed, which are also the subject of the present invention, advantageously carry such salt tolerant trait. Plants may be regenerated from transformed cells to yield transgenic plants, which may be screened for certain levels of salt tolerance. In a preferred embodiment, the exogenous nucleic acid encodes AVP1, or a homologue thereof. Preferably expression of the vacuolar pyrophosphatase in the plant is enhanced to an extent that the transgenic plant is tolerant to sodium chloride (NaCl) when the NaCl concentration is from about 0.2M to about 0.3M. A transgenic plant capable of growing in salt water may also be produced by introducing into one or more cells of a plant nucleic acid which upregulates expression of vacuolar pyrophosphatase in the plant to yield transformed cells. As used herein, "salt water" includes water characterized by the presence of salt, and preferably wherein the concentration of salt in the water is from about 0.2M to about 0.4M. In one embodiment, salt water refers to sea water.

The transgenic plants of the present invention can also be used to produce double transgenic plants which are tolerant to salt (about 0.2M to about 0.4M salt concentration). In one embodiment, the present invention relates to a double transgenic plant which is tolerant to salt comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of a vacuolar pyrophosphatase and an  $\text{Na}^+/\text{H}^+$  antiporter in the plant. The vacuolar pyrophosphatase in an advantageous construct is AVP1, or a homologue thereof, and the  $\text{Na}^+/\text{H}^+$  antiporter is AtNHX1, or a homologue thereof. The present invention also encompasses transgenic progeny of the double transgenic plant, as well as seeds produced by the transgenic plant and a progeny transgenic plant grown from the seed.

Drought and/or freeze tolerance may also be introduced into plants by transforming plant cells with exogenous nucleic acid which alters the expression of vacuolar pyrophosphatase in the plant such that such expression is upregulated. In a preferred embodiment there is provided a substantially drought and/or freeze resistant transgenic plant which comprises a genome having one or more exogenously introduced vacuolar  $\text{H}^+$ -translocating pump genes. A particularly preferred fertile transgenic plant eliciting drought and/or freeze tolerance, as well as the ability to grow in saline soils, comprises an isolated exogenous chimeric DNA construct encoding vacuolar  $\text{H}^+$ -translocating pump, preferably operably linked to a promoter, such as the 35-S promoter or any other strong promoter, including, without limitation, tissue specific promoters. The



transgenic plant may contain a polynucleotide sequence comprising an exogenous tonoplast pyrophosphate  $H^+$  pump gene operably linked to a promoter. In yet another particularly preferred drought and/or freeze resistant transgenic plant having the capacity to grow in saline soils, the polynucleotide sequence comprises an exogenous tonoplast pyrophosphate  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter. A particularly preferred tonoplast pyrophosphate  $H^+$  pump gene is the AVP1 gene.

Upregulation of expression of vacuolar pyrophosphatase by the methods described above may also be used to provide a plant having larger vegetative and/or sexual organs than wild type counterpart plants. That is, the present invention provides for a method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant which is larger than its corresponding wild type plant. Also encompassed by the present invention is a method of making a transgenic plant (*e.g.*, an ornamental plant) having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells.

There is also disclosed in the present invention novel gene cassettes including cassettes comprising a tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a chimeric promoter, a novel gene cassette comprising a exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a promoter, as well as novel coding sequences comprising an exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter. Preferably such coding sequence is designed to overexpress AVP1.

There is also disclosed in the present invention novel expression vectors including an expression vector containing a polynucleotide sequence comprising a exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter and further operatively linked to a multiple cloning site, and an expression vector containing a polynucleotide sequence comprising a

exogenous tonoplast pyrophosphate driven  $H^+$  pump gene operably linked to a double tandem enhancer of the 35S promoter and further operatively linked to a heterologous coding sequence.

It is recognized by the present inventor, that the disclosed invention may have application to any plant, including, without limitation, crop plants, ornamental plants, grasses, shrubs, or any other plant found useful or pleasing to man.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

The above description, as well as further objects, features and advantages of the present invention will be more fully understood with reference to the following detailed description when taken in conjunction with the accompanying drawings, wherein:

*Fig. 1A* is an overhead view of representative (out of 10 plants each) wild type (WT) and two independent transgenic lines (1' and 2') grown hydroponically for seven weeks on a 10 hour light/dark cycle;

*Figs. 1B(1), 1B(2) and 1B(3)* are a photomicrographs of the root and root hairs of representative five day old seedlings obtained from representative WT, 1' and 2' of *Fig. 1A* grown parallel to the surface on vertical plant nutrient agar plates;

*Fig. 1C* is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (1' and 2') overexpressing AVP-1;

*Fig. 2* is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') after exposure to 7 days of water deficit stress.

*Fig. 3* is a perspective view of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil.

*Fig. 4A* is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment; Nhx1 ( $Na^+/H^+$  antiporter), Vmal (vacuolar membrane  $H^+$ -ATPase), Gef1 (yeast CLC chloride channel), Ena1 (plasma membrane  $Na^+$ -ATPase).

*Fig. 4B* is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment shown in *Fig. 4A*, which also includes Avp1 (*A. thaliana* vacuolar pyrophosphate-energized proton pump).

*Fig. 5A* and *Fig. 5B* are bar graphs showing the intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type yeast strains and of yeast strains carrying various mutations affecting sodium tolerance wherein the values are the mean of two determinations, and the bars represent the standard deviations.

*Figs. 6A, 6B, 6C*, when joined by matchlines A-A and B-B, is an alignment of the deduced amino acid sequences of NhX1 homologue from Arabidopsis *AtNHX1* (SEQ ID NO:1), human *HsNHE-6* (SEQ ID NO: 2) and yeast *ScNHX1* (SEQ ID NO:3); identical residues are in black boxes, and dashes indicate gaps in the sequence, \* above alignment denote putative amiloride binding site from human *NHE1* ( $^{163}\text{DVF-FLFLLPPI}^{173}$ ) (SEQ ID NO: 4).

*Fig. 7* is a bar graph of  $\text{Na}^+$  and  $\text{K}^+$  content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil.

*Fig. 8* is a graph of the uptake of calcium into the 35SAVP-1 transgenic vacuolar membrane vesicles (squares) of 2' of *Fig. 3* versus calcium uptake into vesicles obtained from wild type (WT) of *Fig. 3*.

*Figs. 9A and 9B* are illustrations demonstrating the theorized mechanism for a higher accumulation of solids into vacuoles via a proton driven function versus that of WT vacuoles.

#### **DETAILED DESCRIPTION OF THE INVENTION**

Since plant vacuoles constitute 40 to 99% of the total intracellular volume of a mature plant cell, changes in the size of the vacuole have dramatic effects upon cell size (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole*. (Academic Press Limited, 1997), vol. 25, pp. 298-337). The volume of the vacuole is controlled by ion and water fluxes mediated by pumps and transporters. In plants the driving force that triggers the movement of ions, solutes and water across membranes is a proton gradient. The activity of the vacuolar  $\text{H}^+$ -pumps results in luminal acidification and the establishment of a  $\text{H}^+$  electrochemical potential gradient across the vacuolar membrane, which powers the secondary active transporters of inorganic ions, sugars, and organic acids. The activity of these transporters modulates cellular pH and ion homeostasis and leads to the accumulation of solutes required to generate the osmotic potential that promotes vacuolar expansion (H. Sze, X. Li, M. G. Palmgren, *The Plant Cell* **11**, 677-689 (1999)).

There are three distinct pumps that generate proton electrochemical gradients. One at the plasma membrane that extrudes  $H^+$  from the cell (PM  $H^+$ -ATPase) and two at the vacuolar membrane or other endomembrane compartments that acidify their lumen (the vacuolar type  $H^+$ -ATPase and  $H^+$ -PPase) (R. A. Leigh, in *The Plant Vacuole* L. a. Sanders, Ed. (Academic Press, San Diego, California, 1997), vol. 25, pp. 171-194.).

Previous work has shown that a decrease in the levels of the A subunit of the vacuolar  $H^+$ -ATPase of carrot, using an antisense construct, resulted in a plant with reduced cell expansion and altered leaf morphology (J. P. Gogarten, et al., *The Plant Cell* 4, 851-864 (1992)). The present inventor has hypothesized that an increased supply of  $H^+$  into the vacuole could cause cell expansion. Recently, based on the theory that as the availability of protons in the vacuolar function of ion accumulation, it has been hypothesized by the same inventor that accumulation of solids in the vacuoles might be useful to protect against draught and to provide for a more freeze resistant plants.

The present inventor has recognized that plants have a number of vacuolar  $H^+$ -translocating pumps, and that by upregulating their activity, increasing their expression, upregulating their transcription and/or translation, or increasing their copy number that one can increase accumulation of solids in the vacuole due to an increase in the availability of protons in the vacuoles. The inventor tested this hypothesis by increasing the copy number of the vacuolar  $H^+$ -translocating pump, the inorganic pyrophosphatase or V-PPase that consists of a single polypeptide (R. G. Zhen, E. J. Kim, P. A. Rea, in *The Plant Vacuole* . (Academic Press Limited, 1997), vol. 25, pp. 298-337). In *Arabidopsis* the V-PPase encoded by the *AVP-1* gene is capable of generating a  $H^+$  gradient across the vacuole membrane (tonoplast) similar in magnitude to that of the vacuolar  $H^+$ -ATPase (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* 89, 1775-1779 (1992)). As would be understood by one of ordinary skill in the art, similar genes in other plants should function in a similar manner.

In one embodiment, a construct comprising a vacuolar pyrophosphatase gene operably linked to a promoter designed to overexpress the vacuolar pyrophosphatase (e.g., an expression cassette) is used to produce the transgenic plants of the present invention. As used herein the term "overexpression" refers to greater expression/activity than occurs in the absence of the construct. In a particular embodiment, a construct comprising an AVP1 gene operably linked to a chimeric promoter designed to overexpress

AVP1 is used to produce the transgenic plants of the present invention. More particularly, the present invention relates to a construct wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.

The transgenic plants of the present invention may find utility other than those associated with the food value or ornamental value. For example, the transgenic plants of the present invention may uptake different or more ions than their wild-type counterparts. As discussed below, studies with mutant yeast strains (*ena1*) demonstrates that  $H^+$ -translocating pumps at the vacuole plays an important role in cation detoxification in higher plants (the plant components involved in an intracellular cation detoxification system being identified by complementing salt-sensitive mutants of the budding yeast *Saccharomyces cerevisiae*). Transgenic plants and/or progeny thereof comprising exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant in accord with such studies may be used to bioremediate soil and growth medium. Such plants can be used to remove cations (*e.g.*, monovalent and/or divalent cations) from a medium which can support plant growth (*e.g.*, soil, water). For example, transformed plants of the present invention can be used to remove sodium (Na), lead (Pb), manganese (Mn) and/or calcium (Ca) ions from a medium which supports plant growth.

To demonstrate the effect that an increased supply of  $H^+$  into the vacuole would have on resistance to drought and/or freeze, and tolerance to salt growth, as well as size of the plants, the present inventor generated transgenic plants containing extra copies of a vacuolar proton pump, *AVP-1*.

*Arabidopsis thaliana* plants were transformed with constructs containing the *AVP-1* gene. Transgenic lines containing extra copies of this gene were then isolated. The *AVP-1*, open reading frame was cloned into the Xma1 site of a modified pRT103 [R. Topfer, V. Matzeit, B. Gronenborn, J. Schell and H-H. Steinbiss, *Nucleic acid Research* 15, 5890 (1987)]. This vector contains a tandem repeat of the 35-S promoter. A HindIII fragment containing the 35-S tandem promoter, AVP-1 ORF and the polyadenylation signal was subcloned into the HindIII site of the pPZP212 vector [P. Hajdukiewicz, Z. Svab and P. Maliga, *Plant Molecular Biology* 25, 989-994 (1994)]. Agrobacterium-mediated transformation was performed via vacuum infiltration of flowering *Arabidopsis thaliana* (ecotype *columbia*). Transgenic plants were selected by plating seeds of the transformed plants on plant nutrient agar plates supplemented with 25 mg/liter kanamycin.

Plants were subsequently selected for two generations to identify transgenic plant homozygous for the transgene.

*Fig. 1A* is an overhead picture of representative (out of 10 plants each) wild type (WT) and two independent transgenic lines (1' and 2') grown hydroponically for seven weeks on a 10 hour light/dark cycle. As can be seen in *Fig. 1A*, a visual comparison of transgenic line 2', which expresses the AVP-1 protein at highest level, transgenic line 1', and wild type (WT), demonstrates that the amount of AVP-1 correlates with the size of the plants. The mass of the transgenic plants was found to be greater than that of wild type. The dry weight of the entire transgenic plants, measured after 24 hours at 75°C (n = 4), for transgenic lines 1' and 2' was found to be 1.5 and 3 times greater than that of wild-type (WT).

*Fig. 1B(1), Fig. 1B(2) and Fig. 1B(3)* are photomicrographs (magnification: times 40; bar length on photograph = 2mm) of the root and root hairs of representative five day old seedlings obtained from representative WT, 1' transgenic and 2' transgenic of *Fig. 1A* grown parallel to the surface on vertical plant nutrient agar plates. Seedlings of both transgenic lines 1' and 2' showed root hairs with an average length 40 and 70% larger than wild-type (WT) root hairs (*Fig. 1B*) (Root hair length along the whole root was determined from five members of each set of seedlings. An average of 80 root hairs per plant were measured). The length of the root hairs is correlated with the size of the vacuole, so the increased size of the root hair is likely to result from increased vacuolar volume. This compares with the *Arabidopsis* mutant *rdh3* which has been reported to have reduced vacuolar volume and is a short plant with abnormally short root hairs (M. E. Galway, J. W. J. Heckman, J. W. Schiefelbein, *Planta* **201**, 209-218 (1997)). It is recognized that the increased root structure will have a positive impact on soil erosion, nitrogen fixation in legumes, and will aid in water uptake by the plant.

*Fig. 1C* is an immunoblot of membrane fractions isolated from wild type (WT) and two independent transgenic lines (1' and 2') overexpressing AVP-1. Total membrane fractions were isolated from shoots of eight week old wild type (WT) and AVP-1 transgenic plants (1' and 2') grown in a hydroponic media for 6 weeks. Shoots of plants homogenates were sequentially centrifuged for 15 and 30 min at 8 and 100 kg respectively. The 100kg membrane pellet was re-suspend in 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 1 mM PMSF Protein (10 mg) was separated

on a 10% SDS-PAGE, electroblotted and immunostained with antibodies raised against a KLH-conjugated synthetic peptide corresponding to the putative hydrophilic loop IV of the AVP-1 protein (V. Sarafian, Y. Kim, R. J. Poole, P. A. Rea, *Proc. Natl. Acad. Sci.* **89**, 1775-1779 (1992)). PPase was detected by chemiluminescence. *Fig. 1C* illustrates that the transgenic lines (1' and 2') express AVP-1 protein at higher levels than the wild type (WT) (that is, 1' = 15% and 2' = 50% more than WT).

As wheat that has been deprived of water is rendered more drought tolerant by an increase in cell  $K^+$  content (from 100 mM to 300 mM) (S. Gupta, G. Berkowitz, P. Pier, *Plant Physiol* **89**, 1358-1365 (1989)), it is hypothesized (but the invention is not hereby limited by such theory) that the increased drought resistance of the AVP-1 transgenic plants may be a consequence of their higher vacuolar concentration of potassium that results in a increased water retention capability. Laboratory tests appear to confirm this.

*Fig. 2* is an overhead view of a representative wild type plant (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') after exposure to 7 days of water deficit stress. Wild type and transgenic plants overexpressing *AVP-1* (*Fig 3A*) were tested for drought tolerance (24°C). After 7 days of water deficit stress wild type (WT) plants withered, whereas plants from both *35S AVP-1* transgenic lines (1' and 2') were turgid and alive. Furthermore, when the drought stressed plants were then watered, transgenic plants pursued normal growth, bolted and set seeds, whereas wild type plants died. The relative water content of leaves from wild type and *35S AVP-1* transgenic plants were determined along the water deficit stress, demonstrating increased water retention by the transgenic lines as compared to the WT plants.

While not illustrated in the accompanying illustrations, similar results may be seen with respect to freeze challenge ( $< 0^{\circ}\text{C}$ ) over a 24 hour or more period for a number of plant species. While not limited to such hypothesis, transgenic plants overexpressing AVP-1 (1' and 2') are believed to provide enhanced protection from freeze as compared to wild type (WT) plants due to the higher amounts of cations in the vacuoles. Higher amounts of cations confer a greater osmotic pressure that leads to a greater water retention capability endowing the plants not only with the ability to withstand low soil water potentials, but also providing greater protection from freezing that leads to significant desiccation of the plants.

*Fig. 3* is a perspective view of wild type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil. Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (1' and 2') were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph in *Fig. 3* corresponds to plants at the tenth day in the presence of 300 mM NaCl. *Fig. 3* illustrates that the two AVP-1 plant types (1' and 2') were significantly hardier in salty soil as compared to wild-type plants. The fact that genetically engineered *Arabidopsis thaliana* plants that overexpress either *AVP1* (the pyrophosphate-energized vacuolar membrane proton pump, this work) or *AtNHX1* (the  $\text{Na}^+/\text{H}^+$  antiporter, (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999)) and this work) are capable of growing in the presence of high NaCl concentrations strongly supports the strategy described herein. A double transgenic plant would be expected to demonstrate a further enhanced salt-tolerant phenotype. These *Arabidopsis thaliana* transporters or their counterparts may perform similar function in important agricultural crops. The increased size of 35S *AVP1 Arabidopsis* transgenic plants also contribute to potential yield increases in genetically engineered crops.

#### **A Working Model of Cation Homeostasis in Plant Organelles**

While the present invention is not limited to any particular hypothesis, the present inventors have developed a working model for cation homeostasis in plant organelles which may explain the unexpected results discovered with respect to the transgenic plants disclosed herein.

In plants, most of the transport processes are energized by the primary translocation of protons.  $\text{H}^+$ -translocating pumps located at the plasma membrane and tonoplast translocated  $\text{H}^+$  from the cytosol to extracellular and vacuolar compartments, respectively (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). The plant tonoplast contains two  $\text{H}^+$ -translocating pumps; the V-ATPase and the inorganic pyrophosphatase or V-PPase. Their action results in luminal acidification and the establishment of a  $\text{H}^+$  electrochemical potential gradient across the tonoplast (Davies, J.M., *et al.*, The Bioenergetics of Vacuolar  $\text{H}^+$  Pumps. In: *Plant*



*Vacuole*, pp. 340-363, Leigh, R.A., Sanders, D. (eds.), Academic Press, San Diego (1997)). The vacuolar membrane is implicated in a broad spectrum of physiological processes that include cytosolic pH stasis, compartmentation of regulatory  $\text{Ca}^{2+}$ , sequestration of toxic ions such as  $\text{Na}^+$ , turgor regulation, and nutrient storage and retrieval. The vacuole constitute 40 to 99% of the total intracellular volume of a mature plant cell. The vacuolar proton pumping pyrophosphatase is a universal and abundant component of plant tonoplast capable of generating a steady-state trans-tonoplast  $\text{H}^+$  electrochemical potential similar or greater than the one generated by the V-ATPase (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). Pyrophosphate (PPi) is a by-product in the activation or polymerization steps of a wide range of biosynthetic pathways and in plants serves as an alternative energy donor to ATP for sucrose mobilization via sucrose synthetase, for glycolysis via PPI: fructose-6-phosphate phosphotransferase and for noplast energization via the vacuolar proton pumping pyrophosphatase (Stitt, M., *Bot. Acta* 111:167-175 (1998)).

Most of intracellular organelles, including clathrin-coated vesicles, endosomes, Golgi membranes and vacuoles have acidic interiors (Xie, X. S., *et al.*, *J. Biol. Chem.*, 264:18870-18873 (1989)). This acidification is mediated by a proton-translocating electrogenic ATPase and in plant vacuoles also via a pyrophosphate-driven proton pump V-PPase (Davies, J.M., *et al.*, The Bioenergetics of Vacuolar  $\text{H}^+$  Pumps. In: Leigh R.A., Sanders, D., (eds) *The Plant Vacuole*, pp. 340-363, Academic Press, San Diego (1997); Zhen, R.G., *et al.*, "The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane Academic Press Limited (1997)). There exists a requirement of anion transport to maintain net electroneutrality (al-Awqati, A., *Curr. Opin. Cell. Biol.*, 7:504-508 (1995)).

Fig. 4A is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment; Nhx1 ( $\text{Na}^+/\text{H}^+$  antiporter), Vmal (vacuolar membrane  $\text{H}^+$ -ATPase), Gef1 (yeast CLC chloride channel), Ena1 (plasma membrane  $\text{Na}^+$ -ATPase). The yeast member of the CLC voltage-gated chloride channel superfamily, Gef1, is required for copper loading in late-Golgi vesicles and for cation sequestration in the pre-vacuolar compartment in yeast (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998); Gaxiola, R.A., *et*

*al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999); Example 1). Furthermore, it has been shown that the defects of *gef1* mutants can be suppressed by the introduction of the prototype member of the CLC superfamily, the *Torpedo marmorata* *CLC-0* or by the introduction of *Arabidopsis thaliana* *CLC-c* and *CLC-d* chloride channel genes (Hechenberger, M., *et al.*, *J. Biol. Chem.*, 271:33632-33638 (1996); Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)). *Fig. 4B* is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast pre-vacuolar compartment shown in Figure 4A, which also includes *Avp1* (*A. thaliana* vacuolar pyrophosphate-energized proton pump).

While not wishing to be bound by theory, two observations led to the proposal of the model for  $\text{Na}^+$  sequestration in yeast illustrated in *Figs. 4A and 4B*. First, *gef1* mutants are sensitive to high NaCl concentrations. Second, the  $\text{Na}^+/\text{H}^+$  exchanger *Nhx1* is localized to the pre-vacuolar compartment (Nass, R., *et al.*, *J. Biol. Chem.*, 273:21054-21060 (1998)). The model proposed in *Figs. 4A and 4B* posits that  $\text{Na}^+$  sequestration by *Nhx1* depends on the vacuolar  $\text{H}^+$ -ATPase and *Gef1*, the chloride channel. *Gef1*-mediated anion influx allows the establishment by the vacuolar  $\text{H}^+$ -ATPase of a proton gradient sufficient in magnitude to drive the uphill accumulation of  $\text{Na}^+$  via  $\text{Na}^+/\text{H}^+$  exchange.

Based on such models, it was theorized that increasing the influx of protons into the postulated endosomal compartment should improve  $\text{Na}^+$  sequestration via the *Nhx1* exchanger. In order to increase the  $\text{H}^+$  availability the *A. thaliana* gain-of-function mutant gene *AVPI-D* that codes for the vacuolar pyrophosphate-energized proton pump was expressed (Figure 3B) (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). This plant pump expressed in yeast restored the  $\text{Na}^+$  resistance of the test strain, but only if the strain had functional *NHX1* and *GEF1* genes. Furthermore, *Gef1p* and *Nhx1p* co-localize within a common organelle, the pre-vacuolar compartment (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). These results strongly support the model in *Figs. 4A and 4B* and indicate that the yeast pre-vacuolar compartment can be used to identify the elusive plant transporters involved intracellular sodium detoxification.

The model set forth in *Figs. 4A and 4B* is entirely consistent with the physiological data on the role of the vacuole in cation detoxification in higher plants. Yeast and plant cells share pathways and signals for the trafficking of vesicles from the

Golgi network to the vacuole (Neuhaus, J.M., *et al.*, *Plant Mol. Biol.*, 38:127-144 (1998); (Paris, N., *et al.*, *Plant Physiol.*, 115:29-39 (1997); Sato, M.H., *et al.*, *J. Biol. Chem.*, 272:24530-24535 (1997); Vitale, A.V., *et al.*, *Trends Plant Sci.*, 4:148-154 (1999)).

Studies were therefore undertaken in yeast to identify the role of the vacuole in cation detoxification in higher plants.

### **Studies of Cation Sequestration Mechanism in Yeast**

#### **Example 1: Functionality of AtNhxl and Avp1 in Yeast Strains**

To test the sequestration models set forth in *Figs. 4A and 4B*, mutant yeast strains (*enal*) lacking the plasma membrane sodium efflux pump, which therefore must rely on the internal detoxification system in order to grow on high salt, were constructed. The sequestration model (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998) and, Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)) of *Figs. 4A and 4B* predicts that the *enal* strain would become salt tolerant if one could enhance the availability of protons in the postulated endosomal compartment, that is with increased influx of protons, cytoplasmic Na<sup>+</sup> would be sequestered via the Nhxl exchanger.

The yeast vacuolar ATPase is a multisubunit protein, so it is difficult to increase its activity by overexpressing any one of its subunits. Instead the same effect was achieved by increasing the influx of protons by expressing the *A. thaliana AVP1* gene in yeast. This gene encodes a single polypeptide that, when expressed in yeast, is capable of pumping protons into the lumen of the vacuole (Kim, E.J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:6128-6132 (1994)). To ensure maximum activity of this proton pump, the E229D gain-of-function mutant of the *AVP1* gene (*AVP1-D*) that has enhanced H<sup>+</sup> pumping capability was expressed (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). Intracellular sodium and potassium content was determined for mutant and wild-type cells after growth on SD-ura medium with high NaCl content.

#### **Materials and Methods**

##### **Yeast strains and Plasmids**

Strains isogenic to W303 (*ura3-1 can1-100 leu2-3, 112trp1-1 his3-11*, (Gaxiola, R.A., *et al.*, *EMBO J.*, 11:3157-3164 (1992)) were employed. Plasmids pRG52 (-*gef1::HIS3*) (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)) and pRG197 (-*nhx1::HIS3*) were used to construct the deletions of *GEF1* and *NHX1*

genes, yielding strains RGY85 and RGY296, respectively. The *enal::HIS3* mutant was obtained from Fink Lab collection (L5709).

#### Method of Transformation

Transformation of yeast cells was performed by using the lithium acetate method (Gietz, D., *et al.*, *Nucleic Acids Res.*, 20:1425 (1992)). Double mutants RGY324 (*gef1::HIS3 enal::HIS3*), RGY326 (*nhx1::HIS3 enal::HIS3*), and RGY343 (*gef1::HIS3 nhx1::HIS3*) were obtained by crossing the single-mutant strains. Double mutants were identified among the meiotic progeny by scoring for the phenotypes associated with each of the single mutants. Sporulation, tetrad dissection, and mating types were scored as described (Guthrie C. and Fink, G.R., *Guide to Yeast Genetics and Molecular Biology* (Academic, San Diego (1991)). Cells were grown in YPD (1% yeast/2%peptone/2% dextrose; Difco), YPGAL (1% yeast/2%peptone/2% galactose; Difco), SD (Difco; Synthetic medium with 2% Dextrose), or APG (APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM MgSO<sub>4</sub>, 1 mM KCl, 0.2 mM CaCl<sub>2</sub>, and trace minerals and vitamins) (Rodriguez-Navarro, A. and Ramos, J., *J. Bacteriol.*, 159:940-945 (1984)). MnCl<sub>2</sub> (Sigma), tetramethylammonium chloride (Sigma), NaCl (Sigma), or hygromycin-B (Sigma) were added as indicated.

Wild type, L5709 (*enal::HIS3*), RGY324 (*gef1::HIS3 enal::HIS3*), and RGY326 (*nhx1::HIS3 enal::HIS3*) strains were transformed with pYES2 vector (Invitrogen) and plasmid pYES2-*AVP1*-E229D described in Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997). The strain RGY343 (*gef1::HIS3 nhx1::HIS3*), used for histochemical analysis, was transformed with pRG151 (*GEF1-GFP*) (Gaxiola, R.A., *et al. Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)) and with pRIN73 [*NHX1*-(HA)<sub>3</sub>] (Nass, R., and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)).

Wild-type and RGY296 (*nhx1::HIS3*) strains were transformed with vector pAD4 (Ballester, R., *et al.*, *Cell*, 59:681-686 (1989)). RGY296 (*nhx1::HIS3*) was transformed with pRG308 (*ADH1::AtNHX1*) (see *Cloning of AtNHX1*).

#### Determination of Intracellular Sodium and Potassium Content

Cells were grown overnight in SD-ura medium (Difco; synthetic medium with 2% dextrose without uracil). YPGAL (1% yeast extract/2% peptone/2% galactose; Difco) media was inoculated with the overnight stocks and grow to an A<sub>600</sub> of 0.6. At this OD, NaCl was added to a final concentration of 0.7 M. The cells were incubated for 6 h,

harvested by centrifugation, washed two times with 1.1 M sorbitol and 20 mM MgCl<sub>2</sub>, and entracted with water for 30 min at 95°C.

The amount of Na<sup>+</sup> and K<sup>+</sup> in cells was determined at the University of Georgia Chemical Analysis Laboratory by an Inductively Coupled Plasma-MS (see world wide web at [rserv/uga.edu/rsnew/chemicalanalysis/](http://rserv/uga.edu/rsnew/chemicalanalysis/)). Intracellular cation concentrations were estimated as described (Gaxiola, R.A., *et al.*, *EMBO J.*, 11:3157-3164 (1992)) by using the intracellular water value calculated for cells grown in 1M NaCl.

#### Immunofluorescence

The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in SD-ura, -leu medium (Difco; synthetic medium with 2% dextrose without uracil and leucin) to mid-logarithmic phase, 0.1 mg/ml hygromycin B was added, and the culture was incubated for 1 h at 30°C. Cells were fixed with 3.7% formaldehyde (Sigma) for 45 min at room temperature without agitation. Spheroplast formation, permeablization, washing, and antibody incubation was performed as described (Pringle, J., *et al.*, in *Immunofluorescence Methods for Yeast*, eds. Guthrie, C. And Fink, G.F. (Academic, Sand Diego), Vol. 194 pp.565-602 (1991)). MAB HA11 used as first antibody was from Babco (Richmond, CA). Cy3-conjugated goat anti-mouse IgG was from Jackson Immunoresearch. 4',6-Diamidino-2-phenylindole (Sigma) was added to mounting medium to stain mitochondrial and nuclear DNA.

#### Subcellular Fractionation and Western Analysis

The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in APG medium (pH 7.0), and lysates fractionated on a 10-step sucrose density gradient as described (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Aliquots of individual fractions (100 µg) were subjected to SDS/PAGE and transferred to nitrocellulose as described (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Western blots were probed with monoclonal anti-GFP (green fluorescent protein) antibody (1:10,000 dilution; CLONTECH), anti-hemagglutinin antibody (1:10,000 dilution: Boehringer Mannheim), and peroxidase-coupled goat anti-mouse antibody (1:5,000;) and developed by using the ECL enhanced chemiluminescence system (Amersham Pharmacia).

#### Cloning of *AtNHX1*

*AtNHX1* was cloned from a phage cDNA library of *A. thaliana* (Kieber, J.J., *et al.*, *Cell*, 72:427-441 (1993)) (obtained from the Arabidopsis Biological Resource

Center) by probing with an expressed sequence tag (Arabidopsis Biological Resources Center, DNA Stock Center) containing a partial clone. A full-length clone (2.1 kB) was ligated into vector pSK2 (Stratagene) at the *NotI* sit, generating plasmid pRG293. The *AtNHX1* ORF was amplified via PCR by using pRG293 as template and GGCCCGGGATGGATTCTCTAGTGTCGAAACTGCCTTCG (SEQ ID NO: 5) (italicized bases correspond to nucleotides 1-30 of the ORF) and T7 oligonucleotides. The PCR product was then digested with *XbaI* and *SalI* and ligated into pAD4 vector generating plasmid pRG308. The *AtNHX1* ORF was sequenced to verify the fidelity of the PCR product. The full-length sequence is longer than the ORF reported by the Arabidopsis Genome Initiative (A TM021B04.4), and has been deposited in GenBank (accession no. AF106324).

#### Cloning of AVP1-D

Vector pYES2 (Invitrogen) was introduced into wild-type, *enal*, *enal nhx1*, and *enal gef1* mutants. Plasmid pYes2-AVP1-D (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)) was introduced into *enal*, *enal nhx1*, and *enal gef1* mutants. Five-fold serial dilutions (starting at  $10^5$  cells) of each strain were plated on YPGAL (1% yeast extract/2% peptone/2% galactose) with or without 0.5 M NaCl and incubated at 30°C for 2 days. Exponentially growing cells (wild-type and *enal* transformed with pYES2 vector and *enal*, *enal nhx1*, and *enal gef1* mutants carrying pYes2-AVP1-D) were exposed to 0.7M NaCl for 6 hours. Total cell extracts were prepared, and  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined.

#### Results

The *enal* mutant of the above construct lacks the plasma membrane sodium efflux pump and therefore must rely on the internal detoxification system to overcome sodium toxicity. Growth of the *enal* strain is sensitive to low concentrations of sodium (200 mM), concentrations that do not inhibit the growth of wild-type strains. Overexpression of AVP1-D restored salt tolerance to salt-sensitive *enal* mutants. The restoration of salt tolerance to an *enal* strain by AVP1-D requires functional *NHX1* and *GEF1* genes: *enalnhx1 AVP1-D* and *enal gef1 AVP1-D* strains are salt sensitive.

*Fig. 5A* and *Fig. 5B* are bar graphs showing the intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type yeast strains and of yeast strains carrying various mutations affecting sodium tolerance wherein the values are the mean of two determinations, and the bars

represent the standard deviations. The intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents of wild-type strains and of strains carrying various mutations affecting sodium tolerance were determined after 6 h of exposure to media supplemented with 0.7 M NaCl. The intracellular  $\text{Na}^+$  content in the *enal* mutant was seen to be 8-fold higher than in the wild-type strain. There was seen to be a consistent reduction in total cell  $\text{Na}^+$  in the *enal AVP-D* strain. The reason for this reduction is unknown. The *enal AVP-D* strain was found to be salt-resistant, even though its intracellular  $\text{Na}^+$  content was 4-fold higher than that of the wild type. In *enal AVP1-D* strains lacking either *gef1* or *nhx1* (i.e., *enal gef1* or *enal nhx1*), the  $\text{Na}^+$  content was not reduced to the extent that it was in the *GEF1 NHX1* strain. Taken together, the genetic and physiological data are consistent with the model that *Nhx1*, *Gef1* and *Avp1* cooperate to sequester sodium internally. As can be seen in the graphs, the *Arabidopsis* Vacuolar  $\text{H}^+$ -Pyrophosphatase (*Avp1*) was evidenced to confer salt tolerance to yeast *enal* mutants.

The intracellular  $\text{K}^+$  content was found to correlate with salt tolerance and is inversely correlated with the  $\text{Na}^+$  content of the strains (*Fig. 4B*). The wild-type  $\text{K}^+$  concentration was at 100 mM, but was reduced to 20 mM in the *enal* mutant. Interestingly, in an *enal* strain that overexpresses the *AVP1-D* gene, the intracellular concentration of  $\text{K}^+$  was restored almost to wild-type levels (*Fig. 4B*). However, *AVP1-D* overexpression failed to restore wild-type levels of intracellular potassium unless both *NHx1* and *GEF1* were functional (See, the double mutants *enal nhx1* or *enal gef1* in *Fig. 4B*).

As shown herein, intracellular  $\text{Na}^+$  detoxification in yeast requires functional  $\text{Na}^+/\text{H}^+$  exchanger (*Nhx1*) and chloride channel (*Gef1*), and they co-localize to a pre-vacuolar compartment (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). When the *Arabidopsis thaliana* homologue of the yeast *NHX1* gene (*AtNHX1*) was cloned and its function in the *nhx1* yeast mutant tested, the *AtNHX1* gene was found to be able to suppress partially the cation sensitivity phenotypes of *nhx1* mutants. *Fig. 6* is alignment of the deduced amino acid sequences of *NhX1* homologue from *Arabidopsis AtNHX1* (SEQ ID NO:1), human *HsNHE-6* (SEQ ID NO: 2) and yeast *ScNHX1* (SEQ ID NO:3); identical residues are in black boxes, and dashes indicate gaps in the sequence, \* above alignment denote putative amiloride binding site from human *NHE1* ( $^{163}\text{DVF-FLFLPPI}^{173}$ ) (SEQ ID NO: 4).

Example 2: Functionality of , and Co-localization of, Gef1p and Nhx1p in Yeast Strains

The *NHX1* and *GEF1* genes, which have been identified as important in sodium detoxification, are also required for the detoxification of other cations. An investigation was made with respect to the viability of yeast strains mutant with respect to *gef1* and *nhx1* (*enal*) in the presence of toxic cations, in light of the model set forth in Figs. 4A and 4B.

The sequestration model postulates not only a functional connection between the anion channel Gef1 and sodium exchanger Nhx1 but also predicts that these two proteins co-localize within a common compartment. Because previous studies indicated that Nhx1 localizes to a pre-vacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)), experiments were also performed to determine whether Gef1 and Nhx1 proteins co-localize to this compartment.

Materials and Methods

The strain RGY419 (*gef1 nhx1*) was transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-(HA)<sub>3</sub>. Transformants were grown in SD (Difco; synthetic medium with 2% dextrose). To determine the sensitivity of such transformants to toxic cations, five-fold serial dilutions (starting at 10<sup>5</sup> cells) of the indicated strains were grown at 30 °C for 2 days on YPD (1% yeast extract/2% peptone/2% dextrose) with the addition of either 3 mM MnCl<sub>2</sub>, 0.45 M tetramethylammonium (TMA), or 0.05 mg/ml hygromycin B (HYG) as indicated.

Two studies were undertaken to demonstrate co-localization of *Gef1p* and *Nhx1p*.

Distribution of fluorescence and immunodetection of subcellular fractions in *gef1 nhx1* cells transformed with two constructs: a *GEF1-GFP* fusion and a *NHX1-(HA)<sub>3</sub>*-tagged fusion were determined. When the cells reached OD<sub>600</sub> = 0.5, hygromycin B (Sigma) was added to a final concentration of 0.1 mg/ml and the cells were incubated for 40 min at 30 °C. Cells were fixed and stained with antibodies to HA epitope and 4',6-diamidino-2-phenylindole (DAPI). Cells were viewed by charge-coupled device microscopy and optically sectioned by using a deconvolution algorithm (Scanalytics, Billerica, MA) (Kennedy, B.K., *et al.*, *Cell*, 89:381-391 (1997)); (Bar = 1 μm.).

The migration properties of the *Gef1p* and *Nhx1p* in sucrose gradients was also determined to provide evidence of co-localization of Nhx1 (HA)<sub>3</sub> and GEF1-GFP.



The strain RGY419 (*gef1 nhx1*) was transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-(HA)<sub>3</sub> and grown in APG medium (Rodriguez-Navarro, A. and Rea, P.A., *J. Biol. Chem.*, 159:940-945 (1984)). Such was converted to spheroplasts, lysed, and fractionated on a 10-step sucrose gradient (18-54%) as described (Sorin, A., *et al.*, *J. Biol. Chem.*, 272:9895-9901 (1997) and Antebi, A. and Fink, G.R., *Mol. Biol. Cell*, 3:633-654 (1992)). Western blots showed the distribution of Gef1-GFP and Nhx1-HA.

### Results

*Gef1* mutants were found to be sensitive to 3 mM MnCl<sub>2</sub>, 0.45 M tetramethylammonium chloride and to 0.05 g/ml hygromycin-B. The *nhx1* mutant was also found to be sensitive to tetramethylammonium chloride and hygromycin. The extreme sensitivity of the *nhx1* mutant to hygromycin may provide an important tool for assaying nhx1 function.

It was found that hemagglutinin (HA)-tagged Nhx1 and Gef1-GFP fusion protein co-localize as shown via epifluorescence deconvolution microscope. Persistence of signal coincidence on 90° rotation of the image further supports co-localization of the two transporter proteins in these cells. The co-localization of Nhx1 (HA)<sub>3</sub> and GEF1-GFP is also supported by the co-migration of the two proteins in sucrose density gradients of membrane preparations obtained from cells expressing the tagged proteins. The sedimentation behavior of the membrane fraction containing both proteins is consistent with that of a pre-vacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). Gef1-GFP (but not Nhx1) is also present in Golgi fractions, consistent with previous studies (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998), Schwappach, B., *et al.*, *J. Biol. Chem.*, 273:15110-15118 (1998)).

### Example 3: Capacity of *A. thaliana* Homolog of NHX1 to Suppress Hygromycin Sensitivity of Mutant Yeast

The yeast strain described herein provides an important tool for identifying genes that mediate salt tolerance in other organisms. To test the utility of this system, a sequence from *Arabidopsis* (See *Materials and Methods*) with very high homology to the *S. cerevisiae* NHX1 ORF was identified and used an expressed sequence tag (see *Materials and Methods*) to obtain a full-length clone of this *Arabidopsis* gene. An alignment of the amino acid sequences of Nhx1 homologues from *Arabidopsis* (AtNhx1),

human (HsNhe6), and yeast (ScNhx1) reveals segments of amino acid identity and similarity within predicted transmembrane domains (*Fig. 6A-C*). However, it is important to note that despite these relationships, neither the C-terminal regions of AtNhx1 and ScNhx1 show a high degree of homology (*Fig. 6A-C*).

A characteristic of mammalian  $\text{Na}^+/\text{H}^+$  antiporters is their inhibition by amiloride. A putative amiloride binding site ( $^{163}\text{DVFFFLFLLPPI}^{173}$ ) (SEQ ID NO: 4) has been defined via point mutants in the human NHE1 antiporter gene (Counillon, L., *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:4508-4512 (1993)). AtNhx1, HsNhe-6 and ScNhx1 have an almost identical sequence (*Fig. 6*). However, attempts to inhibit the activity of either Nhx1 or AtNhx1 in yeast cultures with amiloride were unsuccessful.

The extreme sensitivity of yeast *nhx1* mutants to hygromycin permitted the testing of whether the cloned *Arabidopsis AtNHX1* ORF could provide  $\text{Na}^+/\text{H}^+$  exchange function in yeast. Vector pAD4 (Ballester, R., *et al.*, *Cell*, 59:681-686 (1989)) was introduced into wild-type and *nhx1* strains. Plasmid pRG308; ADH; *AtNHX1* was introduced into *nhx1* mutants as indicated. Five-fold serial dilutions (starting at  $10^5$  cells) of the indicated strains were grown at 30 °C for 2 days on YPD (-) or on YPD supplemented with 0.05 mg/ml hygromycin (+). Serial dilutions of the same strains were grown on APG medium (see Materials and Methods) (-) or on APG supplemented with 0.4 M NaCl (Rodriguez-Navarro, A. and Ramos, J., *J. Bacteriol.*, 159:940-945 (1984)).

The *At NHX1* gene is capable of suppressing the hygromycin sensitivity of the *nhx1* mutant. The *AtNHX1* gene also suppressed the NaCl sensitivity of *nhx1* mutant but only under conditions in which the  $\text{K}^+$  availability was reduced. However, *AtNHX1* was not capable of rescuing the  $\text{Na}^+$  sensitive growth phenotype of the double mutant *enal nhx1* overexpressing the *AVP1-D* gene.

#### Example 4: Generation of Gain-of-Function Yeast AtNHX Mutants

##### Materials and Methods

Gain of function mutants of the AtNHX that enhance salt tolerance were generated in the *enal* yeast by mutagenizing the cloned gene to make a mutant library. This library was used to transform the salt sensitive yeast mutant *enal* and clones with an enhanced salt tolerant phenotype.

Other genes that show similarity to the AtNHX1 gene, as reported by the Arabidopsis Genome Initiative (AGI), were also expressed in the mutant yeast to form gain-of-function mutants. It is believed that some of these AtNHX1 homologues are plasma membrane transporters, so their function in yeast are frequently pH dependent, requiring precise composition and pH of the medium used for screening for success. Identification of plasma membrane transporters helps to engineer plants with an enhanced salt tolerance due to a reduced sodium uptake. In addition, plant cDNA expression libraries in yeast are used to identify other families of transporters involved in NaCl detoxification.

To generate gain of function mutants of the AtNHX a method for introducing random mutations developed by Stratgene (Epicurian Coli XL1-Red competent Cells Cat#200129) was used. The method involves the propagation of a cloned gene into a strain deficient in the three primary DNA repair pathways. The random mutation rate in this strain is about 5000-fold higher than that of wild-type. A library of the mutated AtNHX gene was transformed into the *enal* yeast mutant and screened for salt tolerance. Yeast transformation was performed as described by Schiestl and coworkers (Gietz, D., *et al.*, *Nucl. Acid Res.* 20:1425 (1992), incorporated by reference in its entirety herein). An alternative to the XL1-Red random mutagenesis strategy is a PCR approach described by Fink and coworkers (Madhani, H.D., *et al.*, *Cell*, 91:673-684 (1997)).

To test AtNHX1 homologues the same strains and conditions used for AtNHX1 (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)) were used initially. However, if these screening strains and/or conditions did not work, new ones are worked out. When dealing with plasma-membrane AtNHX1 homologues pH conditions of the assay media were found to be crucial.

### Results

The overexpression of the *A. Thaliana* gain-of-function mutant gene *AVP1-D* increases the intracellular detoxification capability in yeast (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). It is hypothesized (although the inventors are not limited by such hypothesis) that the latter is due to an increased influx of H<sup>+</sup> into the vacuolar compartment thereby improving Na<sup>+</sup> sequestration via the Nhx1 exchanger.

### Conclusions from Yeast Cation Sequestration Studies

The yeast studies described above provide evidence for the importance of the pre-vacuolar pH for intracellular  $\text{Na}^+$  sequestration in yeast. Overexpression of the plant  $\text{H}^+$ -pyrophosphatase (Avp1) confers salt tolerance to yeast only in those strains containing a functional chloride channel (Gef1) and the  $\text{Na}^+/\text{H}^+$  exchanger (Nhx1).

These data support a model in which the Nhx1  $\text{Na}^+/\text{H}^+$  exchanger acts in concert with the vacuolar ATPase and the GEF1 anion channel to sequester cations in a pre-vacuolar compartment. Several studies suggest that the pre-vacuolar compartment may be derived both from the plasma membrane and the late Golgi. These vesicles are likely involved in the assembly of the vacuole or delivery of cargo to this organelle. It is reasonable to expect that these pre-vacuolar vesicles detoxify cations by sequestration, thereby lowering their concentrations in the cytoplasm and in other organelles.

The yeast system described herein permits the functional assessment of diverse heterologous proteins in salt tolerance: chloride channels,  $\text{H}^+$  pumps, and  $\text{Na}^+/\text{H}^+$  exchangers and other cation/ $\text{H}^+$  exchangers or cation/bicarbonate symporters. The system is robust and flexible. The function of the *Arabidopsis* chloride channels (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998), Hechenberger, M., *et al.*, *J. Biol. Chem.*, 271:33632-33638 (1996)),  $\text{H}^+$  pump, and  $\text{Na}^+/\text{H}^+$  exchanger can be assayed in the corresponding yeast mutant. Despite the inability of *At NHX1* to suppress all the phenotypes of the yeast *nhx1* mutant, the fact that it suppresses some phenotypes, coupled with the DNA homology between *AtNHX1* and yeast *NHX1*, indicates that the plant gene carries out a similar function to that of the yeast homologue. The observation that the *AtNHX1* gene suppresses the sensitivity of the *nhx1* mutant to hygromycin but provides only a weak  $\text{Na}^+$  detoxification phenotype could be a consequence either of differential regulation of the transporters in the two organisms or of distinct cation transport selectivities.

The regulation of *AtNHX1* by salt and the ability of the plant gene to suppress the yeast *nhx1* mutant suggest that the mechanism by which cations are detoxified in yeast and plants may be similar. Indeed, previous work suggested that vacuolar sodium accumulation in salt-tolerant plants may be mediated by a tonoplast  $\text{Na}^+/\text{H}^+$  antiporter that utilizes the proton-motive force generated by the vacuolar  $\text{H}^+$ -ATPase (V-ATPase) and/or  $\text{H}^+$ -translocating pyrophosphatase (V-Ppase; refs. Barkla,

B.J., *et al.*, *Symp. Soc. Exp. Biol.*, 48:141-153 (1994), Zhen, R.G., *et al.*, *The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane* (Academic, San Diego), Kirsh, M., *et al.*, *Plant Mol. Biol.*, 32:543-547 (1996)).

The finding described herein that both *gef1* and *nhx1* mutants are hypersensitive to hygromycin indicate that the level of resistance to hygromycin depends on the function of the vacuolar and pre-vacuolar organelles. Yeast mutants impaired in K<sup>+</sup> uptake (*trk1*) are hypersensitive to hygromycin (Madrid, R., *et al.*, *J. Biol. Chem.*, 273:14838-14844 (1998)); reduced K<sup>+</sup> uptake hyperpolarizes the plasma membrane potential and drives the uptake of alkali cations such as hygromycin. Mutations that reduce the H<sup>+</sup> pumping activity of the plasma membrane H<sup>+</sup>-ATPase, Pmal, depolarize the plasma membrane potential and confer resistance to hygromycin (McCusker, J.H., *et al.*, *Mol. Cell. Biol.*, 7:4082-4088 (1987)). Thus, mutants such as *gef1* or *nhx1* that affect the pH or membrane potential of the vacuolar and pre-vacuolar compartments may be expected to affect hygromycin compartmentation.

#### **Transgenic Plants With Upregulated Vacuolar H<sup>+</sup>-translocating Pump Activity**

Further support for the role of the *Arabidopsis AtNHX1* gene in salt homeostasis is provided by the observation that its expression is induced in salt-stressed plants (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). *Arabidopsis thaliana*, in particular, has been used as a host model plant to demonstrate that overexpression of these genes results in salt tolerance in the plant. A recent report shows that the overexpression of *AtNHX1* gene in transgenic *Arabidopsis thaliana* promotes sustained growth in soil watered with 200 mM NaCl plus 1/8 M.S. salts under short-day cycle conditions (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999)). It is worth noting that every addition of 1/8 M.S. salts provides 2.5 mM potassium reducing the stringency of the NaCl stress, and that a short-day cycle reduces oxidative stress.

#### **Example 5: Enhanced Expression of At NHX1 Gene in Salt-Stressed Plants**

##### **Materials and Methods**

*A. thaliana* plants (ecotype Columbia) were grown aseptically on unsupplemented plant nutrient agar without sucrose (Haughn, G.W. and Somerville, C., *Mol. Gen. Genet.*, 204:430-434 (1986)) for 15 days at 19°C and under continuous

illumination. NaCl or KCl was added to a final concentration of 250 mM, and the plants were incubated for 6 h. Total RNA from tissue of salt-treated and untreated plants was isolated (Niyogi, K.K. and Fink, G.R., *Plant Cell*, 4:721-733 (1992)), Hybond-N (Amersham) membranes were hybridized with a <sup>32</sup>P-Labeled DNA probe from plasmid pRG308. Hybridization was performed at 65°C overnight. Washes were performed at 65°C with 0.2% standard saline citrate (SSC)/0.1% SDS (Ausebel, F., *et al.*, *Curr. Protocols in Mol. Biol.* (Wiley, NY) (1988)). 18S probe was used as loading control (Unfried, I., *et al.*, *Nucleic Acids Res.*, 17:7513 (1989)). MACBAS 2.4 program was used to quantify the relative amount of RNA.

### Results

The NaCl stress increased *AtNHX1* mRNA levels 4.2-fold, whereas KCl promoted only a 2.8-fold increase. This increase in mRNA level produced by sodium resembles that described for the yeast *NHX1* gene (Nass, R. and Rao, R., *J. Biol. Chem.*, 273:21054-21060 (1998)). RNA tissue blot hybridized with *AtNHX1*. Ten micrograms of total RNA from 15-day old plants exposed to 250 mM NaCl or KCl for 6 h and a control grown without salt was subjected to electrophoresis on a denaturing formaldehyde gel. The blot was hybridized with a probe internal to *AtNHX1* ORF. An 18S ribosomal probe was used as a loading control.

Example 6: Salt tolerance of Transgenic plants overexpressing *AtNHX1*

### Materials and Methods

Transgenic plants that overexpress the *AtNHX1* were generated using *Agrobacterium*-mediated plant transformation. The transgenic *AtNHX1* was expressed using a double tandem enhancer of the 35S promoter of CaMV (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1987)).

15 wild-type plants and 15 35S*AtNHX1* transgenic were grown on a 12 hours-day cycle for 20 days. During this period plants were watered every 5 days with a diluted nutrient solution (1/8 M.S. salts). 200 mM NaCl was added to the watering solution at day 21 and at day 33 plants were watered with a nutrient solution containing 300 mM NaCl. Plants were photographed 10 days after the last NaCl treatment.

### Results

Transgenic plants were found to be significantly less affected than wild type controls when watered with 300 mM NaCl.

#### Example 7: Salt tolerance of Transgenic plants overexpressing 35SAVP1

##### Materials and Methods

A transgenic *Arabidopsis thaliana* plant was engineered to overexpress the *AVP1* wild-type gene using the double tandem enhancer of the 35S promoter (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1987)). *AVP1* encodes the pyrophosphate-energized vacuolar membrane proton pump from *Arabidopsis* (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). Previous investigations suggest that the *AVP1* gene is present in a single copy in the genome of *Arabidopsis* (Kim, Y., *et al.*, *Plant Physiol.*, 106:375-382 (1994)), however, a sequence homologous, but not identical, to *AVP1* on chromosome one has been tentatively designated as ORF F9K20.2 on BAC F9K20 by the *Arabidopsis* Genome Initiative (AGI).

Transgenic plants that overexpress AVP1 were generated using *Agrobacterium*-mediated plant transformation. The transgenic *AVP1* was expressed using a double tandem enhancer of the 35S promoter of CaMV (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1987)). 15 wild-type plants and 15 35SAVP1 transgenics were grown on a 24 hours-day cycle for 16 days. During this period plants were watered every 4 days with a diluted nutrient solution (1/8 M.S. salts). 200 mM NaCl was added to the watering solution at day 17 and at day 27 plants were watered with nutrient solution containing 250 mM NaCl. Plants were photographed 10 days after the last NaCl treatment. Identical conditions and treatment as described in Example 6 were used.

##### Results

Five different lines of 35SAVP1 plants showed an enhanced salt tolerance as compared to wild-type plants in the T2 stage. However, the most dramatic phenotype was apparent in the homozygous T3 plants. These transgenic plants are larger than wild-type plants. Furthermore, homozygous 35SAVP1 plants showed sustained growth in the presence of 250 mM NaCl plus 1/8 M.S. salts when grown in a 24 hours light regimen. When 35SAVP1 plants were grown under short-day cycle conditions (12 hour day/light

cycle) sustained growth in the presence of 300 mM NaCl plus 1/8 M.S. salts was observed.

Example 8: Growth of Wild Type Plants and 35SAVP1 Transgenic Plants in  
Hydroponic Solution

The reduced availability of fresh water for standard agriculture may force the use of alternative agricultural arts. It is conceivable that with salt tolerant crops the use of hydroponics with seawater will create a new era in crop production. Hydroponic culture has been reported to increase plant growth and provide stress-free root and shoot material (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)). Another important advantage of hydroponic culture is that it allows one to alter the ionic composition in a more accurate manner than in soil. These advantages could be important for the physiological studies of salt stress.

Conditions for hydroponics culture of *Arabidopsis* plants were established and their performance in increasing concentrations of NaCl in their media were tested.

Materials and Methods

In one test, hydroponically grown wild type and 35SAVP1 transgenic plants were generated. Wild type and 35SAVP1 transgenic plants were grown in solution culture on a 12 hour light cycle for 65 days.

In another test, wild type and 35SAVP1 transgenic plants were grown in solution culture on a 12 hours light cycle for 20 days. Starting at day 21, NaCl concentration was increased in a stepwise fashion by 50 mM increments every 4 days. Plants were photographed after 4 days in the presence of 200 mM NaCl.

And yet in another hydroponic test, transgenic plants were challenged with a commercial seawater formula that contains the complete ionic composition present in the oceans. 35SAVP1, 35SAAtNHX1 single and double transgenics were grown together with wildtype *Arabidopsis thaliana* plants under hydroponic conditions for four weeks in a short day illumination cycle (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)). Then every four days an equivalent to 50mM NaCl of Tropic Marin sea salt (worldwide web at thatpetplace.com) is added. This artificial sea water mix includes all of the other major and trace elements present in real sea water. Growth was monitored and physiological parameters, such as sodium content and distribution was determined.



### Results

When wild type and 35SAVP1 transgenic plants were grown hydroponically the size differences in root, leaves and stems among wild type and 35SAVP1 transgenic plants were found to be dramatic, with 35SAVP1 transgenic plant parts being much larger.

When NaCl concentration were increased stepwise by 50 mM every 4 days (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999), 35SAVP1 transgenic plants appeared healthy in the presence of 200 mM NaCl while wild type controls showed severe deleterious effects in their leaves and stems.

35SAVP1, 35SAtnHX1 single and double transgenics that were grown together with wildtype *Arabidopsis thaliana* plants under hydroponic conditions for four weeks in a short day illumination cycle (Gibeau, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997)) and then challenged every four days with an equivalent to 50mM NaCl of Tropic Marin sea salt were found to grow in the sea salt solution. Wildtype *Arabidopsis thaliana* plants did not.

#### Example 9: Effect of Overexpression of *Arabidopsis thaliana* Proton Transporters in Tomato Plants

The effects of the overexpression of these *Arabidopsis thaliana* proton transporters (AVP1 and AtNHX1) in a more agriculturally important plant, the tomato plant, was examined. It is believed that increasing the salt-tolerance of tomato plants will likely have important economic repercussions.

The tomato homologues of AVP1 and AtNHX1 were isolated and the corresponding chimeras to overexpress them were constructed (Bidone, S., *et al.*, *Eur. J. Biochem.*, 253: 20-26 (1998); Burbidge, A., *et al.*, *J. Exper. Botany*, 48:2111-2112 (1997)). The genes were introduced via *Agrobacterium*-mediated infection of calli. Tissue culture methods were used to regenerate transformed plants. The plants were assayed for salt tolerance as well as physiological parameters, such as sodium content and distribution.

Tomato transformation with 35S AVP1 and with 35S AtNHX1 constructs was performed as described by McCormick (McCormick, S., Transformation of tomato with *Agrobacterium tumefaciens*. In: *Plant Tissue Culture Manual*, pp. 1-9, Lindsey, K.

(ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands (1991)). T0 and T1 transgenics were analyzed by polymerase chain reaction and DNA gel blotting for the presence and copy number of AVP1 and AtNHX1 transgenes. Heterozygous and homozygous plants were identified after segregation analysis of each transgene within T1 seeds. Homozygous plants were assayed for salt tolerance and as well as physiological parameters, such as sodium content and distribution. Degenerated oligos based on conserved sequences present in AVP1 and AtNHX1 homologues were designed. These degenerated primers were used in RT-PCR reactions with cDNAs made from poly(A)+RNA from tomato. The resulting PCR fragments were used as probes to isolate the full length cDNA clones from commercial libraries (i.e. Stratagene Cat#936004). A similar strategy was described by Caboche and coworkers (Quesada, A., *et al.*, *Plant Mol. Biol.*, 34:265-274 (1997)).

### Results

Positive test results indicate that the sequestration model described herein is also applicable to an important crop.

*Fig. 7* is a graph of  $\text{Na}^+$  and  $\text{K}^+$  content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil. Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (1' and 2') were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph corresponds to plants at the tenth day in the presence of 300 mM NaCl. Parts of the plant above ground were harvested after 24 hours in the presence of 200 mM NaCl and their fresh weight measured. After 48 hours at 75°C, the dry weight was measured.  $\text{Na}^+$  and  $\text{K}^+$  content was determined by atomic absorption. Values in the graphs of *Fig. 4* are the mean  $\pm$  SE ( $n = 4$ ). As can be seen from the graphs  $\text{Na}^+$  and  $\text{K}^+$  content in the transgenic lines (1' and 2') was significantly higher than that of wild-type counterparts.

*Fig. 8* is a graph of the uptake of calcium into the 35SAVP-1 transgenic vacuolar membrane vesicles (squares) of 2' of *Fig. 4* versus calcium uptake into vesicles obtained from wild type (WT) of *Fig. 4*. Wild-type plants (open circles) and transgenic plants from line 2' of *Fig. 4* were grown hydroponically for nine weeks on a 10 hour light

cycle. Vacuolar membrane vesicles were added to buffer containing 250 mM sorbitol, 25 mM BTP-Hepes pH 8.0, 50 mM KCl, 1.5 mM  $\text{MgSO}_4$  and 10  $\mu\text{M}$   $\text{Ca}^{++}$ . This mix was incubated at 20°C for 10 minutes before adding 200  $\mu\text{M}$  PPI to trigger the reaction.  $\text{Ca}^{++}$  ionophore A23187 was added to a final concentration of 5  $\mu\text{g/ml}$  to dissipate the  $\text{Ca}^{++}$  gradient. Aliquot (200  $\mu\text{l}$ ) were filtered at the indicated times and washed with cold buffer as described (11). As is evidenced by the graphs, the transgenic plants from line 2' have greater calcium uptake than wild-type plants.

The above data is consistent with the hypothesis that transgenic plants overexpressing *AVP-1* have an enhanced  $\text{H}^+$  pumping capability at their tonoplast and that an enhanced  $\text{H}^+$  supply results in greater ion accumulation in the vacuole through the action of  $\text{H}^+$ -driven ion transporters. To further support this theory,  $\text{Ca}^{++}$  uptake capability of wild type and transgenic vacuolar membrane vesicles was determined.

It is well documented that  $\text{Ca}^{++}$  enters the plant vacuole via a  $\text{Ca}^{++}/\text{H}^+$  antiporter (K. S. Schumaker, H. Sze, *Plant Physiol.* **79**, 1111-1117 (1985)). Furthermore, the genes encoding the *Arabidopsis thaliana*  $\text{Ca}^{++}/\text{H}^+$  antiporters *CAX1* and *CAX2* have been isolated and characterized (K. D. Hirschi, R.-G. Zhen, K. W. Cunningham, P. A. Rea, G. R. Fink, *Proc. Natl. Acad. Sci. USA* **93**, 8782-8786 (1996)). Fig. 8 shows that  $\text{Ca}^{++}$  uptake in the *35SAVP-1* transgenic vacuolar membrane vesicles is 36% higher than it is in vesicles obtained from wild type. Application of the  $\text{Ca}^{++}$  ionophore A23 lowered the  $^{45}\text{Ca}^{++}$  counts to background levels demonstrating the tightness of the vesicles (Fig 8) (K. S. Schumaker, H. Sze, *Plant Physiol.* **79**, 1111-1117 (1985)).

While not limited by such theory, a model consistent with the enhanced drought and freeze tolerance of the transgenic plants overexpressing the *AVP-1* gene is depicted in Figs. 9A and 9B. The model depicts how an increase in the number of AVP-1 pumps in the vacuole of transgenic plants can provide more  $\text{H}^+$  that will permit the secondary transporters to import greater amounts of cations into the lumen of the vacuoles. Higher amounts of cations confer a greater osmotic pressure that leads to a greater water retention capability endowing plants to withstand low soil water potentials.

Example 10: Double Transgenic Plant with 35S AVP1 and 35S AtNHX1

Overexpression of the pyrophosphate-energized vacuolar membrane proton pump AVP1 likely increases the availability of  $\text{H}^+$  in the lumen of the vacuole, and the

AtNHX1  $\text{Na}^+/\text{H}^+$  antiporter uses these  $\text{H}^+$  to sequester  $\text{Na}^+$  cations into the vacuole. Therefore, higher expression of these transporters likely maximizes the sequestration capability of the vacuole.

To generate transgenic *Arabidopsis* plants that overexpress both genes *AVP1* and *AtNHX1*, T3 35S *AVP1* plants are used as females and T3 35S *AtNHX1* plants are used as males. Female plants are hand-emasculated and anthers from freshly opened flowers of donor plants are harvested. With these anthers the emasculated plants are pollinated by touching the anthers onto the stigmas. The pollinated flowers are labeled and any remaining opened or unopened flowers from the same female plant are removed to avoid any confusion at harvest. The harvested seeds are sterilized using a 50% sodium hypochloride solution and mixed vigorously for 5 minutes and rinsed with water thoroughly. The sterilized seeds are stored in soft agar over night at 4°C. Then they are sprinkled onto solidified kanamycin-hygromycin selective medium. The 35S *AVP1* construct has the neomycin phosphotransferase II gene that confers kanamycin tolerance in plants while the 35S *AtNHX1* construct has a modified hygromycin B phosphotransferase that confers hygromycin tolerance in plants. The resistant seedlings are transplanted into soil and to the hydroponic media to be tested for their salt-tolerant phenotype.

A transgenic *Arabidopsis thaliana* plant to overexpress the *A. thaliana* gain-of-function mutant gene *AVP1-D* (Zhen, *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)) is engineered using the same double tandem enhancer of the 35A promoter described above (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1997)). Plants overexpressing the gain of function mutant gene will likely show an enhanced phenotype. These plants are characterized in parallel with the 35S *AVP1*, 35S *AtNHX* singles and doubles transgenics. The *A. thaliana* gain-of-function mutant gene *AVP1-D* is subcloned into plasmid pRT103 carrying the 35S promoter and the polyadenylation signal of CaMV (Topfer, R., *et al.*, *Nucl. Acid Res.*, 15:5890 (1997)). A HindIII fragment containing the chimeric 35S *AVP-D* gene is subcloned into pBIBhyg (Becker, D., *Nucl. Acid Res.*, 18:203 (1990)). The resulting T-DNA vector is transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al.*, *C.R. Jeances Acad. Sci. Ser.*

*III Sci. Vie*, 316:1194-1199 (1993)). Integration is confirmed on Southern blots of T3 plants and expression monitored on Northern blots of positive T3 plants.

Example 11: Comparative Transport Study With Vacuoles From The Roots Of Wild-Type and 35S AVPI Transgenic Plants

A study was undertaken to determine if the vacuoles of 35S AVPI transgenic plants show a higher proton transport activity dependent on pyrophosphate. These determinations were done with root and shoot tissues separately from plants grown hydroponically. The transgene could show a tissue-specific regulation despite the 35S promoter.

In order to compare the PPI-dependent  $H^+$  translocation activities of wild-type and 35S AVPI transgenic plants sealed tonoplast-enriched vesicles from roots and leaves of the above plants are prepared. The homogenization and differential centrifugation procedure described by Rea and Turner (Rea, P.A., *et al.*, Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In: *Meth. Plant Biochem.*, pp. 385-405, Academic Press limited, London (1990)) is followed.  $H^+$  translocation is assayed fluorimetrically using acridine orange (2.5  $\mu$ M) as transmembrane pH difference indicator in assay media containing vacuole membrane-enriched vesicles as described by Rea and coworkers (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 272:22340-22348 (1997)). The assay media contains 300  $\cdot$  M Tris-PPI, 50mM KCl, 2.5  $\mu$ M acridine orange, 5mM Tris-Mes (pH 8.0). Intravesicular acidification is triggered with the addition of 1.3 mM  $MgSO_4$  and terminated with the addition of the protonophore FCCP at 2.5  $\mu$ M. Fluorescence is measured at excitation emission wavelengths of 495 and 540 nM, respectively, at a slit width of 5 nM (Zhen, R.G., *et al.*, *J. Biol. Chem.*, 269:23342-23350 (1994)). A further test to support that the  $H^+$  translocation is AVPI driven is the addition of the specific inhibitor aminomethylenediphosphonate (Zhen, R.G., *et al.*, *Plant Physiol.*, 104:153-159 (1994)).

Example 12: Determination Of The  $Na^+/K^+$  Ratios In Leaves And Stems Of The Transgenic Plants

Toxic concentrations of NaCl build up first in the fully expanded leaves where NaCl is compartmentalized in the vacuoles. Exposure to NaCl can disrupt or

reduce  $K^+$  uptake leading to  $K^+$  deficiency and growth inhibition (Wu, S.J., *et al.*, *Plant Cell*, 8:617-627 (1996). A cytosolic consequence of reduced  $K^+$  content and high  $Na^+$  is the inhibition of important enzymes. An example of such enzymes is the 3'(2'), 5'-bisphosphate nucleotidase of yeast whose activity is more sensitive to  $Na^+$  when  $K^+$  content is low (Murguia, J.R., *et al.*, *Science*, 267:232-234 (1995).

Measurements may be taken to demonstrate that the transgenic plants described herein have an increased vacuolar capacity to sequester  $Na^+$  in their leaves cells or elsewhere. To determine the  $Na^+/K^+$  ratios in leaves and stem S wild-type and 35S *AVP1* /35S *AtNHX1* double and single transgenics in hydroponic conditions (Gibeaut, D.M., *et al.*, *Plant Physiol.*, 317-319 (1997) are grown. NaCl is added to the growth media in a stepwise fashion starting with 50 mM up to 250mM (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999). At every point the rosette and the stems of the treated plants are collected and their weight is determined. The samples are dried out in an oven at 80°C and their dry weight is determined. The dry samples are boiled in a determined volume of water and their  $Na^+$  and  $K^+$  contents determined via atomic absorption spectrophotometry (Apse, M., *et al.*, *Science*, 285:1256-1258 (1999); Gaxiola, R., *et al.*, *Embo J.*, 11:3157-2164 (1992)).

Example 13: Determination Of Whether 35S *AVP1* Transgenic Plants Are Larger Because Their Cells Are Larger Or Because They Have More Cells, Or Both

The shoot meristems labeling index is compared with one of the wild-type plants. Morphological and anatomical observations measuring and counting cells of leaves, roots and stems are performed. To determine if 35S *AVP1* transgenic plants are larger because they have more cells, their shoot meristems labeling index is compared with the one of wild-type plants.

To measure the DNA synthesis or cell proliferation 5-Bromo-2'-deoxy-uridine (BrdU) that can be incorporated into DNA in place of thymidine may be used. Cells that have incorporated BrdU into DNA are detected using a monoclonal antibody against BrdU monoclonal antibody and an anti-mouse Ig-alkaline phosphatase as a second antibody. The bound anti-BrdU monoclonal antibody is visualized by light microscopy and the ratio between DAPI stained and BrdU positives established. The protocol is a

modification of the one published by Chiatante and coworkers (Levi, M., *et al.*, *Physiol. Plant.* 71:68-72 (1987)) and the BrdU labeling and detection kit II from Boehringer Mannheim. The plants are exposed for different times to the BrdU labeling medium and then fixation, paraffin embedding and sectioning is performed as described by Meyerowitz and coworkers (Drews, G., *et al.*, *Plant Mol. Biol. Rep.*, 5:242-250 (1988)).

For observation of leaf tissue, fresh tissues are embedded in 5% agarose and slice them with a microslicer. For primary root observation, seedlings are fixed for 4hr in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde at room temperature, dehydrate them in graded ethanol series, permeate them with xylene, and infiltrate them with paraffin. Eight-micrometer sections are stained with 0.05% toluidine blue and cells are counted under a microscope. As an alternative for the visualization and determination of cell size the method described by Greenberg and coworkers (Rate, *et al.*, *The Plant Cell*, 11:1695-1708 (1999)) is followed.

#### Example 14: Isolation Of Mutants In The Transporters

Genetic approaches are very powerful in analyzing complex biological traits (Serrano, R., *Crit. Rev. Plant Sci.*, 13:121-138 (1994)) Reverse genetics is a very important new tool for plant biologists. The generation of a good collection of tagged knockouts by Sussman and coworkers (Krysan, P., *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:8145-8150 (1996)) has open a very important avenue for the analysis of gene disruptions in *Arabidopsis*.

The Arabidopsis Knock-out Facility of the University of Wisconsin Madison (world wide web at [biotech.wisc.edu/NewServicesAndResearch/Arabidopsis](http://biotech.wisc.edu/NewServicesAndResearch/Arabidopsis)) is used to search among the 60,480 Arabidopsis (ecotype WS) lines that have been transformed with the T-DNA vector pD991 for the presence of T-DNA inserts within *AtCLC-c*, *AtCLC-d*, *AVP1*, *AtNHX1* and their homologues. The phenotypes of the above knock-outs will shed light towards the understanding of the physiological roles of these transporters in normal and stress conditions. An initial characterization of the knockout plants includes testing for their salt tolerance and their  $\text{Na}^+/\text{K}^+$  ratios. The generation of double knock-outs via crosses help to further understand the interaction among the transporters as well as the crosses with the *35S AVP1* and the *35S AtNHX1* transgenic plants.

To search for *Arabidopsis* knock-out PCR primers are designed following the guidelines detailed in the University of Wisconsin web site. Tested primers are sent to UW-Madison, where 62 PCR reactions that are sent to us for Southern blot analysis are performed. Positive PCR products are sequenced. If the sequence reveals that there is a T-DNA inserted within the gene the gene specific primers are sent for another set of PCR reactions in order to determine which of the 9 possible pools of 225 contains the knockout. After identifying the pool of interest, 25 tubes of seeds are screened for the individual plant carrying the T-DNA knock-out.

Example 15: Cation Detoxification In Plant Cells

The studies described herein together with other evidence strongly indicate that yeast and plants share pathways and signals for the trafficking of vesicles from Golgi network to the vacuole (Gaxiola, R., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999); Marty, F., "The Biogenesis of Vacuoles: Insights from Microscopy. In: *The Plant Vacuole*, 1-42, Leigh, R.A. and Sanders, D., Academic Press, San Diego (1997); Bassham, D.C., *et al.*, *Plant Physiol.*, 117:407-415 (1998)).

Without wishing to be bound by theory, it is believe likely by the present inventor that in both systems a pre-vacuolar compartment is a dynamic entity that detoxifies the cytoplasm from toxic cations and delivers its cargo either to the vacuole, or directly to the cell exterior. Both the *gef1* chloride channel and *Nhx1* Na<sup>+</sup>/H<sup>+</sup> exchanger have been localized to the yeast pre-vacuolar compartment (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999)). The behavior of the *gef1*-GFP chimera in yeast cells in vivo have been monitored indicating that its localization varies depending the environmental conditions. Furthermore, it has been shown that two of the four *A. thaliana* CLC chloride channel genes CLC-*c* and -*d* are capable of suppressing *gef1* mutant phenotypes implying a similar localization (Gaxiola, R.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:4046-4050 (1998)).

In order to understand how and where this cation detoxification takes place in plant cells the intracellular localization of GFP chimeras of AVP1, AtNHX1 and AtCLC-*c* and -*d* (Hong, B., *et al.*, *Plant Physiol.*, 119:1165-1175 (1999)) is monitored *in vivo*. Confocal microscopy is also used to address co-localization of the different transporters. For this purpose HA-tagged versions or antibodies of the transporters under



study are required (Guiltinan, M.J., *et al.*, *Meth. Cell Biol.*, 49:143-151 (1995); Jauh, G. - Y., *et al.*, *Plant Cell*, 11:1867-1882 (1999); Mullen, R.T., *et al.*, *Plant. J.*, 12:313-322 (1997)).

For the constructions of the GFP-chimeras the soluble versions GFP with improved fluorescence in *A. thaliana* reported by Davis and Viestra (Davies, S.J., Viestra, R.D., "Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*, <http://brindabella.mrc-lmb.cam.ac.uk/IndexGFP.html> (1998)) are used. Two types of GFP-chimeras are made, namely a set under the regulation of the native promoter and another set under the regulation of the 35S promoter. The resulting T-DNA vectors containing the GFP-chimeras are transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al.*, *C.R. Jeances Acad. Sci. Ser. III Sci. Vie*, 316:1194-1199 (1993)). For the hemagglutinin (HA) epitope tagging a PCR strategy designed for yeast but modified to tag plant genes expressed in yeast vectors is used. Futcher and coworkers designed vectors containing the URA3 yeast gene flanked by direct repeats of epitope tags (HA) (Schneider, B.L., *et al.*, *Yeast*, 11:1265-1274 (1995)). Via PCR the tag-URA3-tag cassette is amplified such that the resulting PCR fragment possess homology at each end to the gene of interest. *In vivo* recombination in yeast is then used to direct the integration of the PCR-chimera to the plasmid carrying the plant ORF of interest, transformants are selected by the URA<sup>+</sup> phenotype. The URA3 gene is "popped out" when positive transformants are grown in the presence of 5-fluoro-orotic acid. The vector carrying the plant gene has a selection marker different than the URA3 gene.

In conclusion, the manipulation of vacuolar V-PPases in economically important crops could provide an important avenue for crop improvement. Drought and freeze tolerant cultivars could provide new agricultural approaches in areas lost due to drought or minimal rainfall, as well as to provide farmers with protection from unanticipated frosts (freezing rain etc.). Such crops may also be able to be raised on soils considered too saline for wild type crops.

While the invention has been described with respect to preferred embodiments, those skilled in the art will readily appreciate that various changes and/or modifications can be made to the invention without departing from the spirit or scope of

the invention as defined by the appended claims. All references cited in this specification are herein incorporated by reference to the same extent as if each individual reference was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A gene cassette comprising a tonoplast pyrophosphate driven H<sup>+</sup> pump gene operably linked to a chimeric promoter.
2. A gene cassette of claim 1 wherein the tonoplast pyrophosphate driven H<sup>+</sup> pump gene is exogenous.
3. A coding sequence comprising a exogenous tonoplast pyrophosphate driven H<sup>+</sup> pump gene operably linked to a double tandem enhancer of the 35S promoter.
4. An expression vector containing a polynucleotide sequence comprising a exogenous tonoplast pyrophosphate driven H<sup>+</sup> pump gene operably linked to a double tandem enhancer of the 35S promoter and further operatively linked to a multiple cloning site.
5. A drought and freeze resistant transgenic plant comprising a polynucleotide sequence comprising a exogenous tonoplast pyrophosphate driven H<sup>+</sup> pump gene operably linked to a promoter.
6. A seed produced by the drought and freeze resistant transgenic plant of claim 5.
7. A progeny plant from the seed of claim 6.
8. A drought and freeze resistant transgenic plant containing a polynucleotide sequence comprising a exogenous tonoplast pyrophosphate driven H<sup>+</sup> pump gene operably linked to a double tandem enhancer of the 35S promoter.
9. A transgenic plant containing a polynucleotide sequence comprising a multiplicity of exogenous tonoplast pyrophosphate driven H<sup>+</sup> pump genes operably linked to a double tandem enhancer of the 35S promoter, wherein the number of pyrophosphate driven H<sup>+</sup> pump genes is sufficient to express a sufficient number of pyrophosphate driven H<sup>+</sup> pumps on the vacuolar membranes to produce drought and freeze resistance as compared to wild-type plants.
10. A transgenic plant which is tolerant to a salt, comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.
11. The transgenic plant of Claim 10 wherein the exogenous nucleic acid encodes AVP1.

12. The transgenic plant of Claim 11 wherein the exogenous nucleic acid encodes a homologue of AVP1.
13. The transgenic plant of Claim 12 wherein the homologue of AVP1 is obtained from tobacco, bacteria, tomato or corn.
14. The transgenic plant of Claim 12 wherein the AVP1 is present in a construct designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.
15. The transgenic plant of Claim 14 wherein the construct comprises AVP1 operably linked to a double tandem enhancer of a 35S promoter.
16. The transgenic plant of Claim 12 wherein the AVP1 is derived from a wild type plant that corresponds to a wild type of the transgenic plant.
17. The transgenic plant of Claim 12 wherein the AVP1 is derived from a wild type plant that does not correspond to a wild type of the transgenic plant .
18. A transgenic plant which grows in a concentration of a salt that inhibits growth of a corresponding non-transgenic plant.
19. The transgenic plant of Claim 18 wherein the concentration of salt is about 0.2M to about 0.3M.
20. The transgenic plant of Claim 11 wherein the plant is larger than a corresponding non-transgenic plant.
21. A transgenic progeny of the transgenic plant of Claim 11.
22. Seeds produced by the transgenic plant of Claim 11.

23. A progeny transgenic plant grown from seed of Claim 22.
24. A transgenic plant which is tolerant to a salt comprising an exogenous nucleic acid construct which is designed to overexpresses AVP1 or designed to down regulate endogenous pyrophosphatase.
25. A transgenic progeny of the transgenic plant of Claim 24.
26. Seeds produced by the transgenic plant of Claim 24.
27. A progeny transgenic plant grown from seed of Claim 26.
28. The transgenic plant of Claim 14 wherein the construct comprises an AVP1 gene operably linked to a double tandem enhancer of a 35S promoter.
29. A construct comprising an AVP1 gene operably linked to a chimeric promoter designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.
30. The construct of Claim 29 wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.
31. A transgenic plant obtained by introducing into a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.
32. Plant cells comprising exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant cell.
33. The plant cells of Claim 32 wherein the plant cells are root cells or stem cells.
34. The plant cells of Claim 32 wherein the exogenous nucleic acid encodes AVP1.

35. The plant cells of Claim 34 wherein the AVP1 is present in a construct designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.
36. The plant cells of Claim 35 wherein the construct comprises the AVP1 gene operably linked to a chimeric promoter designed to overexpress AVP1.
37. The plant cells of Claim 36 wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.
38. The plant cells of Claim 34 wherein the AVP1 is derived from a wild type plant that corresponds to a wild type of the transgenic plant.
39. The plant cells of Claim 34 wherein the AVP1 is derived from a wild type plant that does not correspond to a wild type of the transgenic plant.
40. A method of making a transgenic plant which is tolerant to a salt comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells in the plant, thereby producing a transgenic plant which is tolerant to the salt.
41. The method of Claim 40 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is tolerant to the salt, thereby producing a transgenic plant which is tolerant to the salt.
42. The method of Claim 40 wherein the exogenous nucleic acid encodes AVP1.
43. The method of Claim 42 wherein the AVP1 is present in a construct designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.

44. The method of Claim 43 wherein the construct comprises the AVP1 gene operably linked to a chimeric promoter designed to overexpress AVP1.
45. The method of Claim 44 wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.
46. The method of Claim 42 wherein the AVP1 is derived from a wild type plant that corresponds to a wild type of the transgenic plant.
47. The method of Claim 42 wherein the AVP1 is derived from a wild type plant that does not correspond to a wild type of the transgenic plant.
48. The method of Claim 40 wherein the plant is tolerant to a concentration of salt that inhibits growth of a corresponding non-transgenic plant.
49. The transgenic plant of Claim 48 wherein the concentration of salt is about 0.2M to about 0.3M.
50. A transgenic plant produced by the method of Claim 40.
51. A method of making a transgenic plant which is tolerant to a salt comprising introducing into one or more cells of a plant a nucleic acid construct which is designed to overexpress AVP1 to yield transformed cells thereby producing a transgenic plant which is tolerant to the salt.
52. The method of Claim 51 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is tolerant to the salt, thereby producing a transgenic plant which is tolerant to the salt.
53. A transgenic plant produced by the method of Claim 51.

54. A method of making a transgenic plant which is larger than its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which is larger than its corresponding wild type plant.
55. The method of Claim 54 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant which is larger than its corresponding wild type plant.
56. The method of Claim 54 wherein the exogenous nucleic acid encodes AVP1.
57. The method of Claim 56 wherein the AVP1 is present in a construct designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.
58. The method of Claim 57 wherein the construct comprises the AVP1 gene operably linked to a chimeric promoter designed to overexpress AVP1.
59. The method of Claim 48 wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.
60. The method of Claim 56 wherein the AVP1 is derived from a wild type plant that corresponds to a wild type of the transgenic plant.
61. The method of Claim 56 wherein the AVP1 is derived from a wild type plant that does not correspond to a wild type of the transgenic plant.
62. The method of Claim 54 wherein the transgenic plant is grown in soil.
63. The method of Claim 54 wherein the transgenic plant is grown hydroponically.

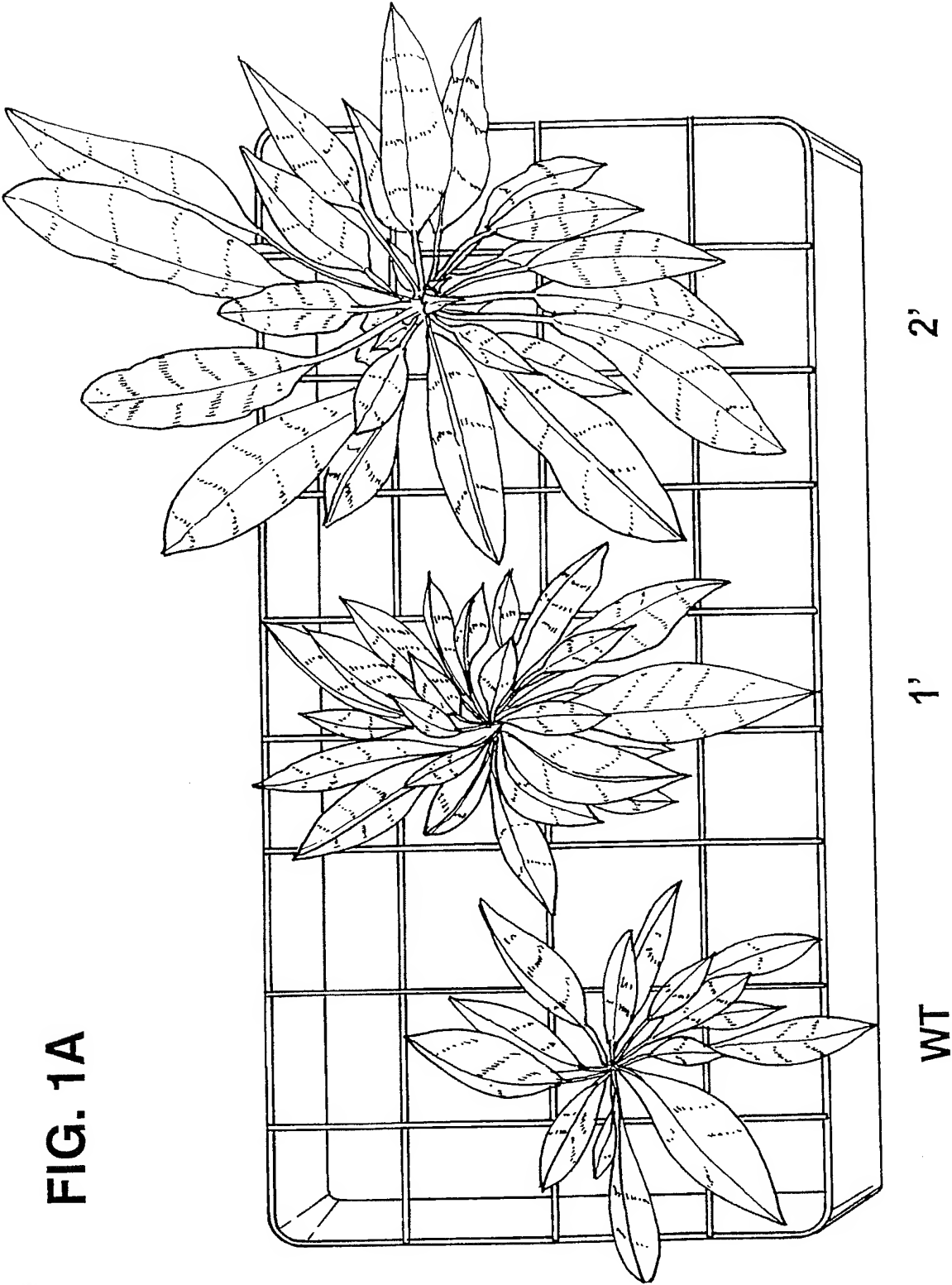


64. A transgenic plant produced by the method of Claim 54.
65. A method of bioremediating soil comprising growing one or more transgenic plants and/or progeny thereof in the soil, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.
66. A method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant.
67. The method of Claim 66 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby increasing the yield of the plant.
68. A method of removing cations from a medium which can support plant growth comprising growing one or more transgenic plants and/or progeny thereof in the medium, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.
69. The method of Claim 68 wherein the medium is selected from the group consisting of: soil and water.
70. The method of Claim 68 wherein the cations are selected from the group consisting of: sodium, calcium, manganese and lead.
71. A method of producing a transgenic plant which grows in salt water comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which can grow in salt water.

72. The method of Claim 71 further comprising regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which grow in salt water, thereby producing a transgenic plant which can grow in salt water.
73. The method of Claim 71 wherein the concentration of the salt water is from about 0.2M to about 0.3M.
74. The method of Claim 73 wherein the salt water is seawater.
75. A transgenic plant which is tolerant to a salt, comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of a vacuolar pyrophosphatase and an  $\text{Na}^+/\text{H}^+$  antiporter in the plant.
76. The transgenic plant of Claim 75 wherein the vacuolar pyrophosphatase is AVP1 or a homologue thereof.
77. The transgenic plant of Claim 76 wherein the homologue of AVP1 is from tobacco, bacteria, tomato or corn.
78. The transgenic plant of Claim 76 wherein the AVP1 is present in a construct designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase.
79. The transgenic plant of Claim 78 wherein the construct comprises AVP1 operably linked to a double tandem enhancer of a 35S promoter.
80. The transgenic plant of Claim 75 wherein the  $\text{Na}^+/\text{H}^+$  antiporter is AtNHX1 or a homologue thereof.
81. A transgenic progeny of the transgenic plant of Claim 75.

- 82. Seeds produced by the transgenic plant of Claim 75.
- 83. A progeny transgenic plant grown from the seed of Claim 72.
- 84. A method of making a transgenic plant having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant having increased flower size compared to its corresponding wild type plant.
- 85. The method of Claim 84 wherein the exogenous nucleic acid encodes AVP1.
- 86. A transgenic plant produced by the method of Claim 84.

FIG. 1A

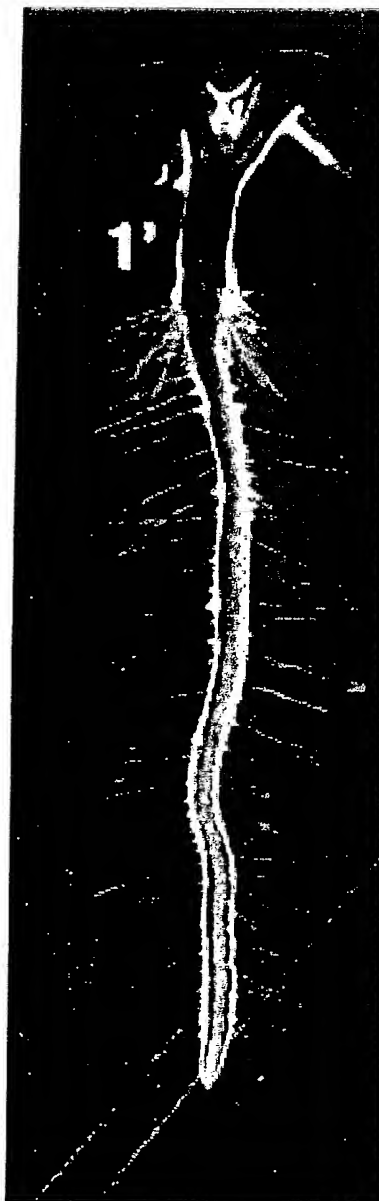


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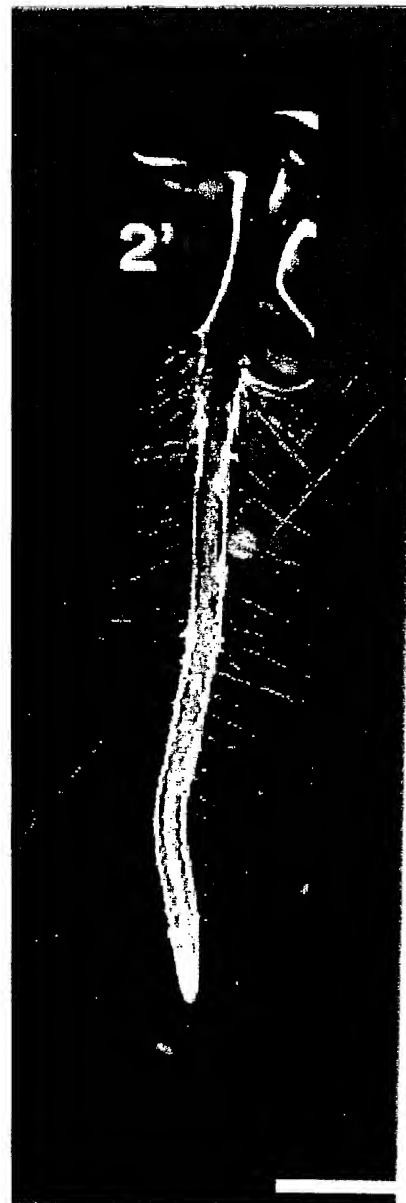
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**FIG. 1B(1)**

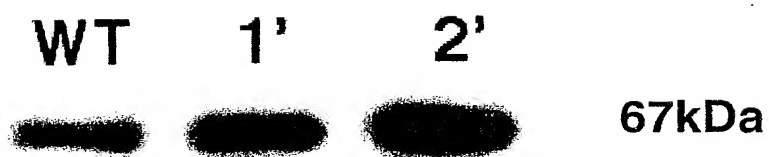


**FIG. 1B(2)**



**FIG. 1B(3)**

3/14



**FIG. 1C**

4/14

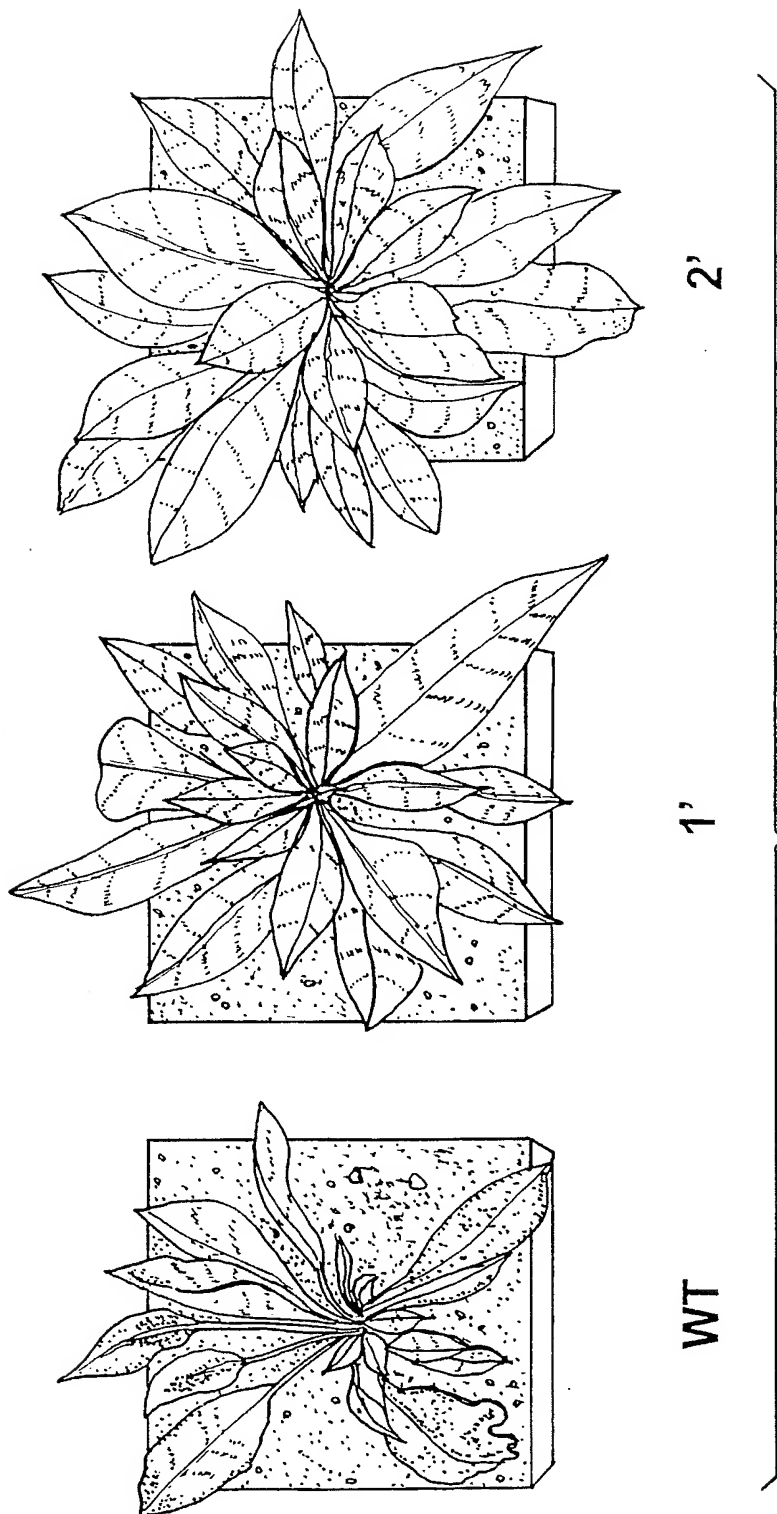
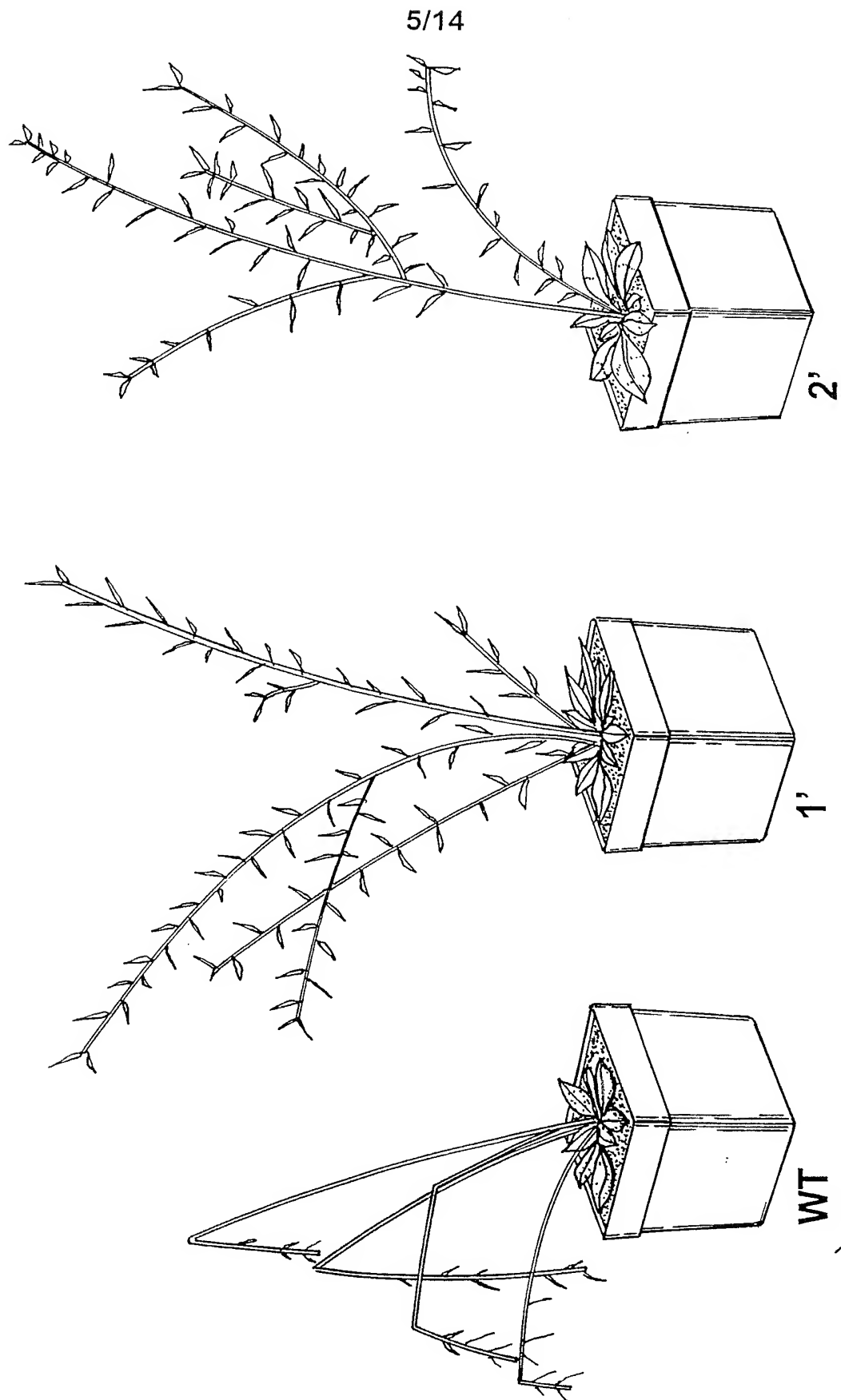


FIG. 2



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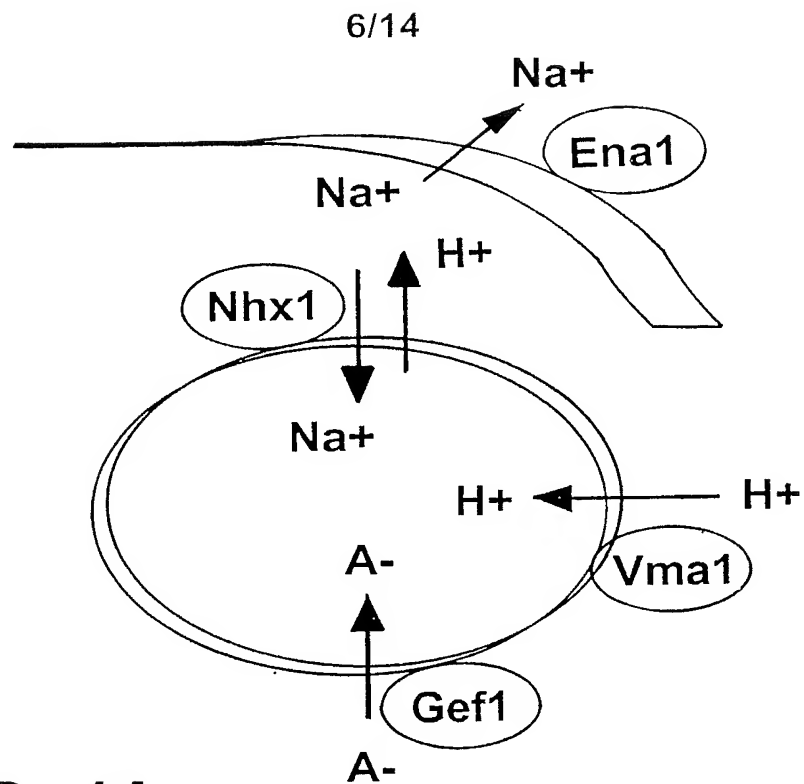


FIG. 4A

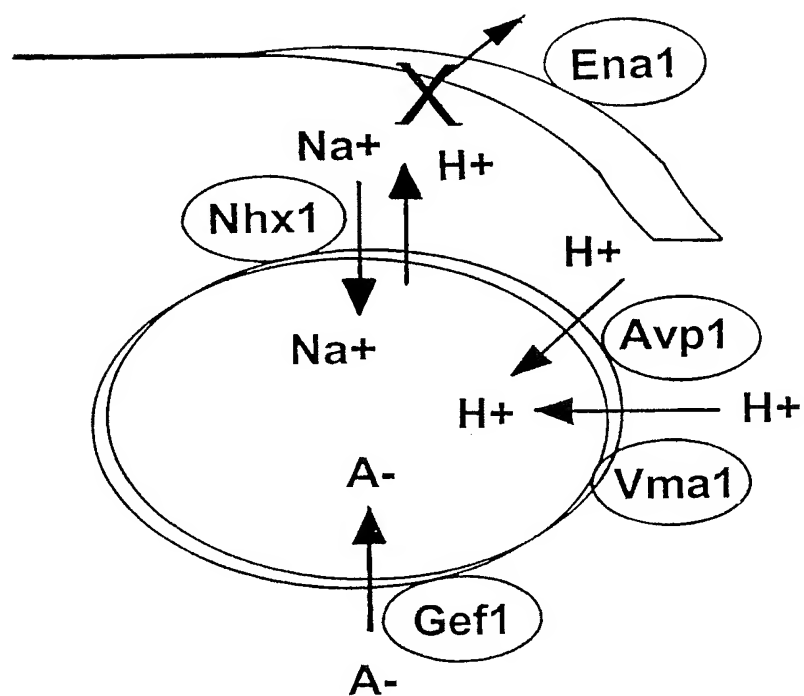
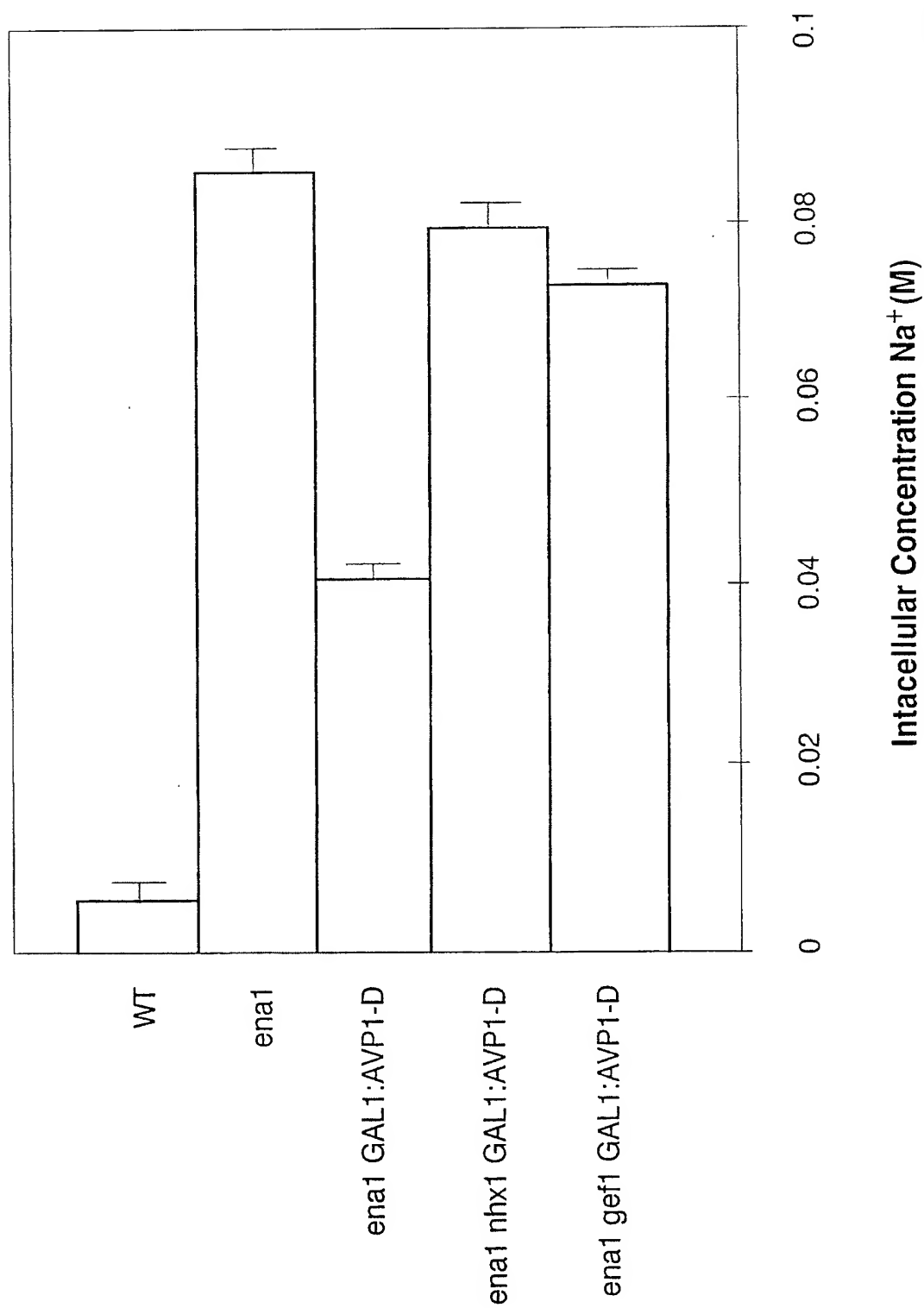
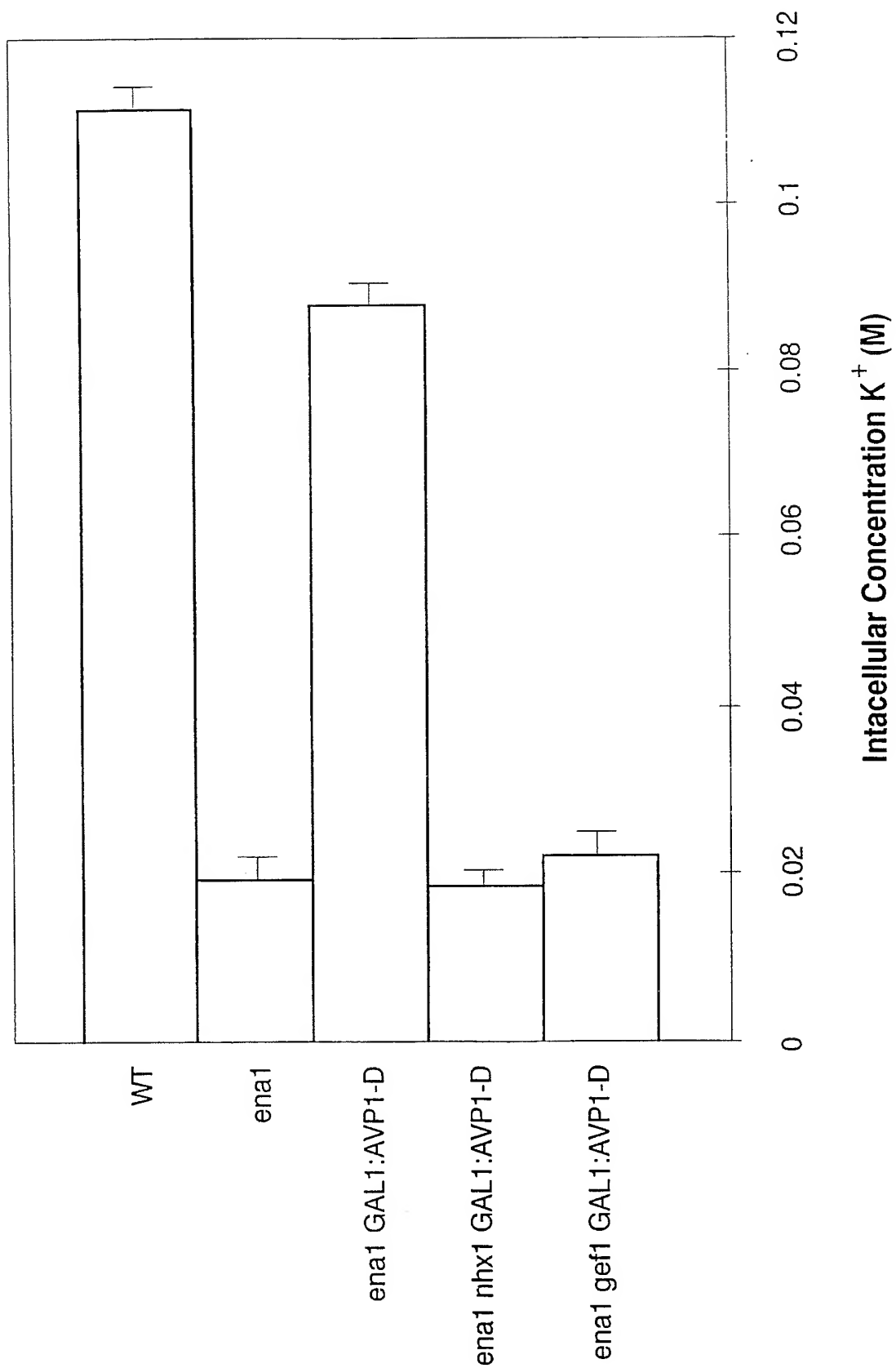


FIG. 4B

7/14

**FIG. 5A**

8/14

**FIG. 5B**

9/14

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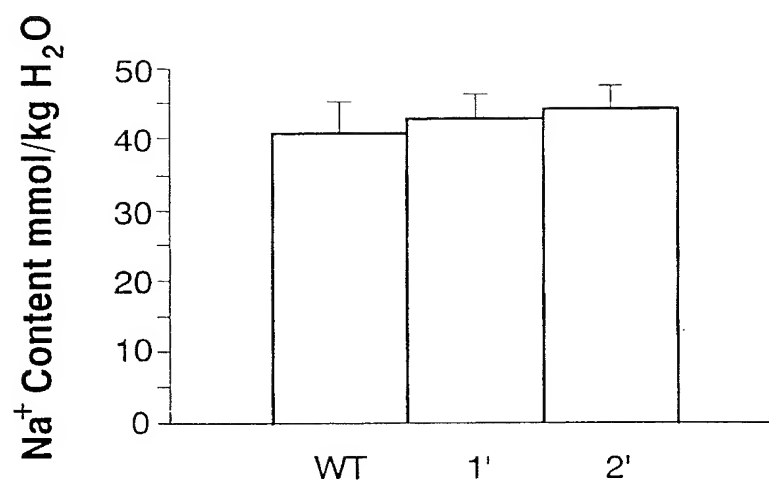
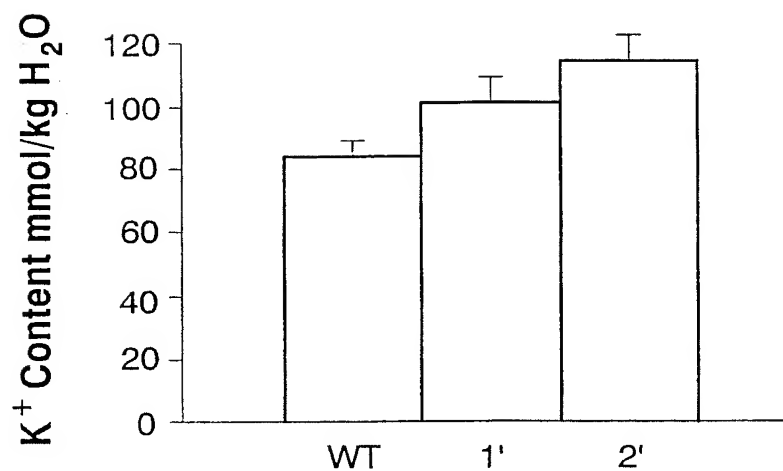
**FIG. 6A**

A		B	
AtNhx1	157	D S V C T L Q V L N Q D E T - P L L Y S L V F C E C V V N D A T S	188
HsNho-6		D P V T V L A I F N E L Q V D P E L Y A L L F C E S V L N D A V A	263
ScNhx1	201	D P V T I L S I F N A Y K V D P K L Y T I I F C E S L L N D A I S	233
AtNhx1	189	V V V F N A I Q S F D L T H - - L - - N H R A A F H L L C N F L	216
HsNho-6	264	I V L S S S I V A Y Q P A C D N S H T F D V T A M F F K S I C I F L	296
ScNhx1	234	I V M F E T C Q K F H C Q P - A T - - F S S - - V F F E C A C L F L	261
AtNhx1	217	Y L F L L S T L L G A A T C L I S A Y V I K K L Y F C R H S T D R	249
HsNho-6	297	G I F S G S F A M G A A T C V V T A L V T K P T K L - R E F Q L L	328
ScNhx1	262	M T F S V S L L I G V L I C I L V A L L K H T H I - R R Y P Q I	
AtNhx1	250	R V A L M M L M A Y L S Y M L A R L F D L S C I L T V P F C G I V	315
HsNho-6	329	R T G L F F L M S W S T F L L A R A W C F T C V V A V L F C G I T	361
ScNhx1	294	R S C L I L L I A Y E S Y F F S N C C H M S C I V S L L F C G I T	326
AtNhx1	283	M S H Y T W H N V T E S S R I T T K H T F A T L S F L A R T F I F	315
HsNho-6	362	Q A H Y T Y N N L S T E S Q H R T T K Q L F H L L N F F I F	384
ScNhx1	327	L K H Y A Y Y N M S R R S Q I T I K Y I F Q L L A R L S F I F	359
AtNhx1	316	L Y V C M D A L D I D K W R S V S D T P G T S I A V S S I L M G L	348
HsNho-6	395	S Y M C L T L F T F Q N H - - V F N P T F V V G A F V A I F L - -	423
ScNhx1	360	I Y L C L E L F T E V E L - - V Y K P L L I I V A I S I C V - -	388
AtNhx1	349	V M V G R A A F V F P L S F L L S N L A K K N Q - - S - - -	372
HsNho-6	424	- - - G R A A N I Y P L S L L L N L G R R S K - - - -	443
ScNhx1	389	- - - A R W C A V F P L S - Q F V N W I Y R V K T I R S M S C I T	417

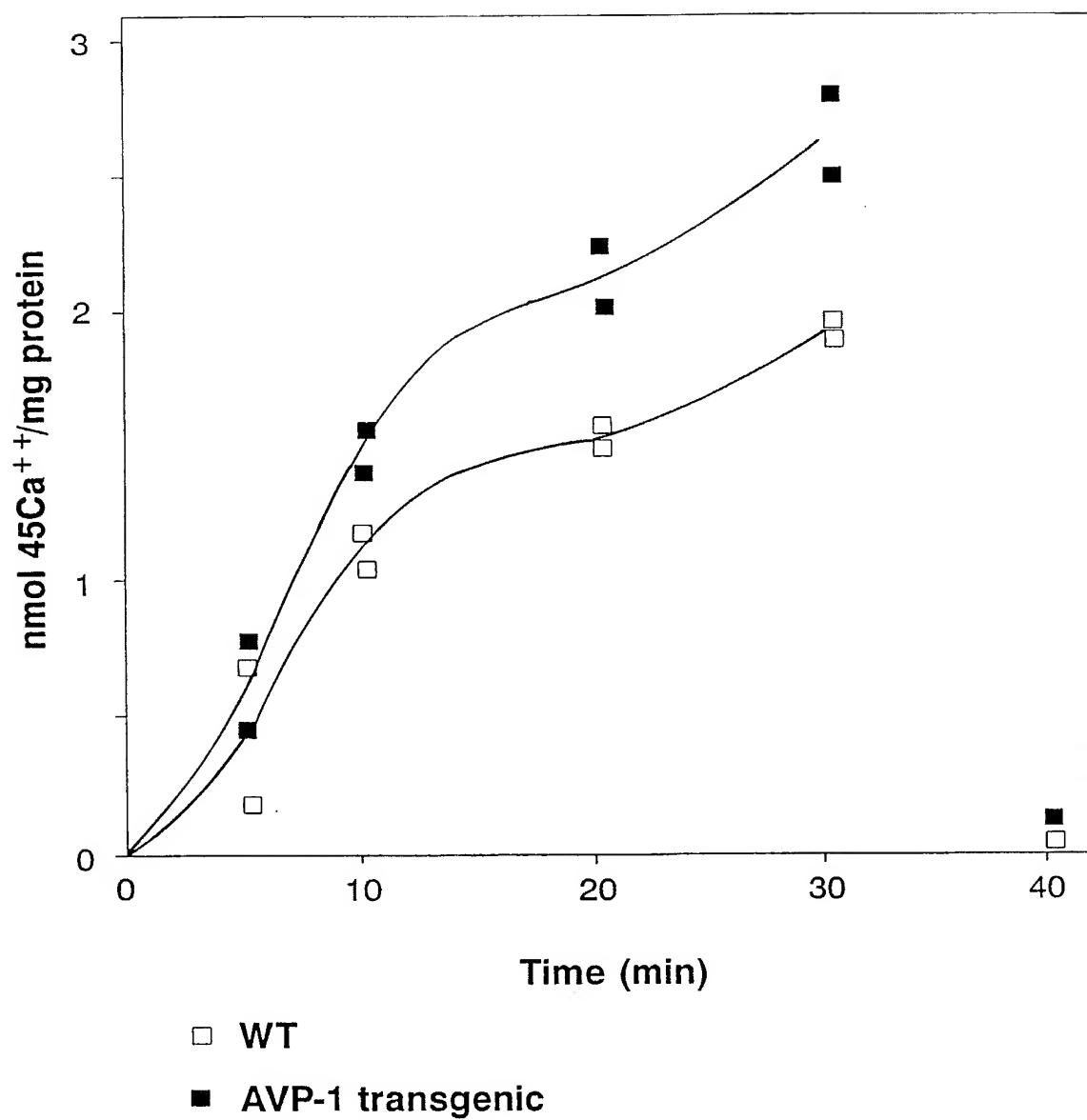
FIG. 6B



12/14

**FIG. 7A****FIG. 7B**

13/14

**FIG. 8**



14/14

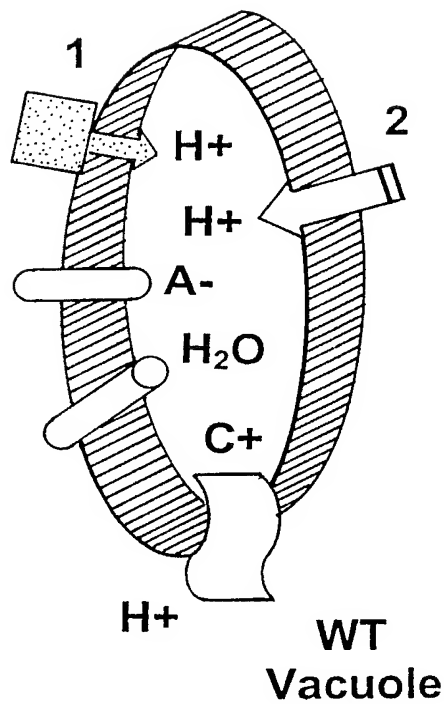


FIG. 9A

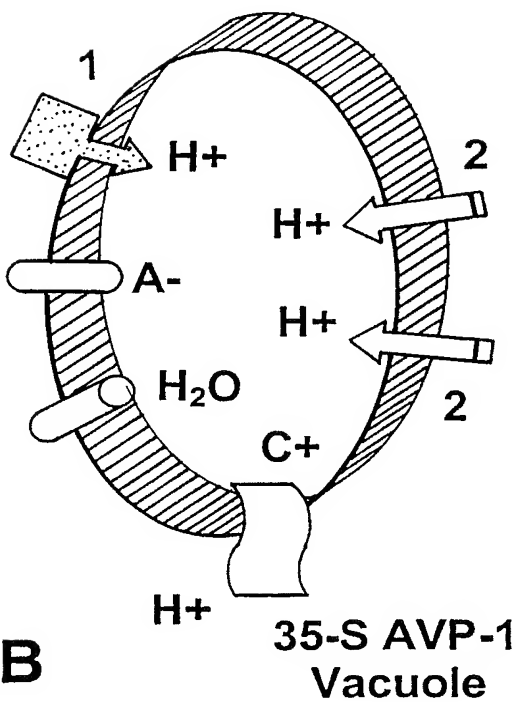


FIG. 9B

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/30955

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A01H 1/00, 5/00; C07H 21/04; C12N 15/00, 15/09, 15/63, 15/70, 15/74, 15/82, 15/87

US CL : 435/320.1; 536/23.6; 800/278, 289, 290, 298

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 536/23.6; 800/278, 289, 290, 298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	APSE et al. Salt tolerance conferred by overexpression of a vacuolar Na <sup>+</sup> /H <sup>+</sup> antiport in Arabidopsis. Science. 20 August 1999, Vol. 285, pages 1256-1258, especially page 1256 abstract and column 3 first full paragraph, page 1257 figure 1 and column 1 last paragraph through column 3, page 1258 figure 3 and column 3 note 15.	10-19, 24-28, 40-53, 68-83
Y	GAXIOLA et al. The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proc. Natl. Acad. Sci. USA. February 1999, Vol. 96, pages 1480-1485, entire document.	10-19, 24-28, 40-53, 68-83
A	DAVIES et al. Vacuolar energization: pumps, shunts and stress. Journal of Experimental Botany. March 1997, Vol. 48, No. 308, pages 633-641.	
A	BALLESTEROS et al. Na <sup>+</sup> /H <sup>+</sup> antiport activity in tonoplast vesicles isolated from sunflower roots induced by NaCl stress. Physiologia Plantarum. 1997, Vol. 99, pages 328-334.	
Y	RAUSCH et al. Salt stress responses of higher plants: the role of proton pumps and Na <sup>+</sup> /H <sup>+</sup> antiporters. J. Plant Physiol. 1996, Volume 148, pages 425-433, especially page 426 column 2 first paragraph through page 427 column 1.	10-19, 24-28, 40-53, 68-83



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P" document published prior to the international filing date but later than the priority date claimed	

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/30955

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SERRANO et al. Microbial models and salt stress tolerance in plants. Critical Reviews in Plant Sciences. 1994, Vol. 13, No. 2, pages 121-138, especially page 121 abstract, pages 132-133 VII Perspectives.	10-19, 24-28, 40-53, 68-83
Y	KIM et al. Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. Proc. Natl. Acad. Sci. USA. June 1994, Vol. 91, pages 6128-6132, entire document.	1-86
X	SARAFIAN et al. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. March 1992, Vol. 89, pages 1775-1779, especially page 1777 figure 2 for X, entire document for Y.	1-4, 29
Y		5-28 and 30-86

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/30955

**Continuation of B. FIELDS SEARCHED Item 3:** WEST, STN (agricola, biosis, biotechno, caba, caplus, embase, esbiobase, medline); search terms: inventors' names, tonoplast, vacuole, pyrophosphate, proton pump, gene, stress tolerance, transgenic plant, AVPI

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1960 (US).

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LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
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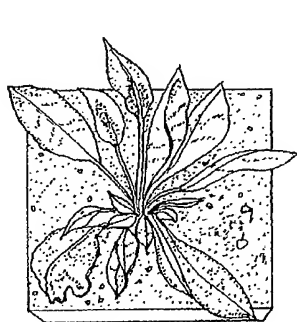
(72) Inventor; and

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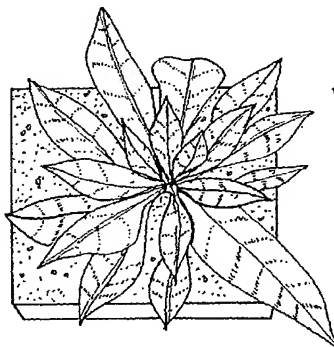
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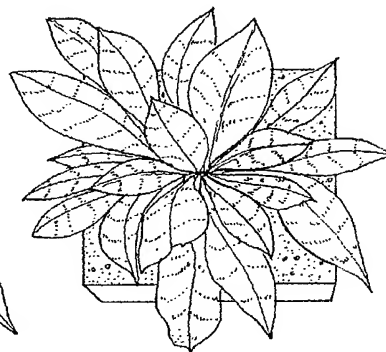
(54) Title: STRESS-RESISTANT OVERSIZED TRANSGENIC PLANTS CAPABLE OF GROWING IN SALINIZED SOIL



WT



1'



2'

(57) Abstract: A stress resistant, oversized, transgenic plant capable of growing in salinized media comprising a polynucleotide sequence causing upregulated express of vacuolar pyrophosphatase. Further disclosed, is the seed produced by such transgenic plants which comprises such polynucleotide sequence, and progeny plants grown from such seed.



WO 01/033945 A1



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